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Evolution of Drug Resistance in *Mycobacterium tuberculosis*

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Abstract

This thesis examines the contrasting roles of genetic drift and selection on the emergence of drug resistance in *Mycobacterium tuberculosis*. In clinical practice some alleles of rifampicin resistance are isolated more frequently than others. To identify if this variation is due to genetic drift or selection, the mutation rate to rifampicin resistance in *M. tuberculosis* (H37Rv) was determined. PCR-SSCP analysis revealed only three patterns from the rifampicin resistant isolates, each pattern arising at the same mutation rate (Mann-Whitney U test $P>0.5$). Fitness, defined as the ratio of generations of resistant and susceptible cells formed in mixed culture, of the differing rifampicin resistant alleles was determined relative to the parent fully susceptible strain. There was a significant correlation between fitness and the clinical isolation rate of each allele (regression analysis $P=0.026$).

The fitness of two isolates, with identical IS6110 RFLP pattern isolated from 2 siblings, was determined. One isolate had developed multi drug resistance, the second isolate had remained fully drug susceptible. The fitness of the drug resistant isolate was significantly lower than the drug susceptible isolate (matched pair t test $p=0.002$). The decreased relative fitness of the resistant isolate implied a physiological cost for the development of drug resistance.

Isolates of *M. tuberculosis* from three patients involved in a hospital outbreak of multi drug resistant tuberculosis were obtained. The fitness of these isolates was determined

relative to H37Rv. Isolates obtained from the same patient did not vary in fitness (one way ANOVA p=0.34). However, the isolates from the three different patients had differing fitness values (one way ANOVA p=<0.001). This implied that there is adaptation of the isolates to the individual patient.

In conclusion, selection has a major role in adaptation of drug resistance in *M. tuberculosis*. This adaptation includes adaptation to the infected host as well as drug resistance.

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Lastly many thanks to my wife Charlotte for her help and support.

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Abbreviations

ADC	Albumin, Dextrose, Catalase solution
AIDS	Acquired immune (or immuno-) deficiency syndrome
Ala	Alanine
Arg	Argenine
Asp	Aspartate
Asn	Asparagine
Bp	Base pairs
CDC	Centre of Disease Control, Atlanta
Cmax	Maximum serum concentration
CFU	Colony forming units
HIV	Human immunodeficiency virus
DNA	Deoxyribonucleic acid
DOTS	Directly observed therapy short course
ED_{50}	The 50% effective dose
G.C ratio	Guanine to Cytosine ratio
Glu	Glutamate
Gly	Glycine
His	Histidine
Leu	Leucine
Lys	Lysine
LJ	Löwenstein Jensen media
OADC	Oleic acid, Albumin, Dextrose, Catalase solution
MBC	Minimum bactericidal concentration
MDR-TB	Multi drug resistant tuberculosis.
MIC	Minimum inhibitory concentration.
MRSA	Methicillin resistant *Staphylococcus aureus*
MTBC	*Mycobacterium tuberculosis* complex
Ne	Effective population size
PAS	Para-aminosalycilic acid
PCR	Polymerase chain reaction

Pro	Proline
PGRS	Polymorphic Guanine cytosine-rich repetitive sequence
PZA	Pyrazinamide
PZAase	Pyrazimidase
RNA	Ribonucleic acid
S.E.	Standard Error
Ser	Serine
SSCP	Single stranded conformation polymorphism
ssDNA	Single stranded deoxyribonucleic acid
tRNA	transfer ribonucleic acid
Trp	Tryptophan
Thr	Threonine
Tyr	Tyrosine
TB	Tuberculosis
UV	Ultra violet light
Val	Valine
WHO	World Health Organisation

Chapter 1

Introduction

1.1 Tuberculosis infection

Tuberculosis (TB) is caused by four species of Mycobacteria: *M. tuberculosis*, *M. africanum*, *M. bovis*, and *M. canettii* (Goh, Legrand, Sola et al. 2001). These four species along with a fifth species *M. microti* form the *Mycobacterium tuberculosis* complex (MTBC). These species are all obligate pathogens, unable to grow in the environment. All five species are genetically homogenous being characterised by 99.9 % similarity at the nucleotide level and identical 16S r RNA sequences (Kapur, Whittam & Musser 1994; Sreevatsan, Pan, Stockbauer et al. 1997).

Species within the MTBC infect different ranges of hosts. *M. microti* is usually associated with voles, however Van Soolingen, Van der Zanden, de Haas et al. (1998) described four human infections with *M. microti*. *M. bovis* infects a range of mammals including ruminants and primates. Human infection with *M. bovis* is more common than with *M. microti* and usually occurs from consumption of infected animal products such as milk and not from person to person infection. *M. tuberculosis M. canettii* and *M. africanum* are pathogens of man and primates with only a significant natural reservoir in humans. *M. tuberculosis* has a substantially diminished virulence for other non-human animal species in comparison to *M. bovis* (Iseman 1994).

M. tuberculosis infection is usually transmitted between people by the inhalation of infected droplets and aerosols produced by infected people coughing and sneezing (Department of Health 1998a). Droplet nuclei of 1 to 5 μm in diameter are the largest particles that can reach the lung alveoli. These particles can only contain 1 to 3 bacilli (Barkley & Kupica 1994). Patients with tuberculosis do produce particles containing more than three bacilli but these are unlikely to reach the lung alveoli of a fresh host and so are less likely to result in infection. This means that fresh infection must arise from these particles of three or less bacteria.

The American Thoracic Society (2000) described four factors determining the likelihood of transmission of infection:

1. the number of organisms being expelled into the air;
2. the concentration of organisms;
3. the length of time an exposed person breathes the contaminated air; and
4. the immune status of the exposed individual.

Human immunodeficiency virus (HIV) infected individuals are more likely to develop infection, however, they are no more likely to transmit infection.

The initial lesion appears as an area of non-specific pneumonitis. It is only after delayed hypersensitivity develops, in 2-4 weeks, that granulomatous inflammation occurs and the characteristic tubercles are formed (Wolinskey 1980). The organisms grow until a

population size of 10^3 to 10^4 bacilli is produced. This size of population stimulates a cellular immune response (Dannenberg, Jr. 1992). In the period before delayed hypersensitivity develops, bacilli are carried to the lymph nodes and then by way of the lymph fluid and infected macrophages in the blood are disseminated throughout the body.

Tuberculosis can occur in any site through out the body. The most common locations for extrapulmonary infection are the genitourinary system, bones and joints, lymph nodes, pleura, and peritoneum. This disseminated tuberculosis can lead to miliary tuberculosis (Wolinskey 1980). Prior to the HIV epidemic, extrapulmonary infection was mainly seen in young children. Pulmonary tuberculosis accounted for approximately 85% of reported cases prior to the HIV epidemic with only 15% of reported cases involving nonpulmonary infection. In HIV infected individuals solely nonpulmonary infection has been reported in 30% of cases, both pulmonary and nonpulmonary infection in 32% of cases and the remaining 38% of cases had solely pulmonary infection (American Thoracic Society 2000).

Primary tuberculosis in the immunocompetant host usually stabilises and heals. Lucas (1989) maintained that haematogenous spread is common in non-vaccinated infected patients, these patients frequently going through a stage in which *M. tuberculosis* is present in the urine indicating infection of the kidney. Disseminated or miliary tuberculosis is, however, an unusual disease and is usually associated with AIDS (Collins 1991).

Caseous lesions heal by fibrosis and calcification. The healed and frequently calcified primary complex lesions are referred to as the Ghon complex, which may be recognised in chest X-rays for the remainder of the infected person's life. In a small proportion of immunocompetent individuals, the infection is not brought under control and the primary lesions become larger, coalesce, and liquefy (Wolinskey 1980). These liquefied cavities enable *M. tuberculosis* to grow extracellularly to produce very large bacterial numbers (Dannenberg, Jr. 1991). When the cavity forms in the lung, liquification can result in the release of vast numbers of bacilli into the respiratory secretions of the infected host. The presence of large numbers of bacilli in sputa is evidence of active pulmonary tuberculosis and the patient is highly infective.

Reactivation of tuberculosis disease can occur decades after the primary infection. The foci are located mostly in the posteria and apical or subapical portions of the lung. By the time the disease is recognised, liquefaction of the caseous lesion has occurred (Wolinskey 1980). This liquefaction, as previously stated, can release vast numbers of bacilli with ready access to the lung.

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1.2 The development of chemotherapy for *M. tuberculosis*

Animal and clinical studies of antitubercular agents have been invaluable in measuring the effectiveness of both the eradication and sterilisation of *M. tuberculosis*. The reduction in colony forming units per day of treatment is an indication of the effectiveness of the killing activity of the antitubercular agent or early bactericidal activity of the agent (Gillespie, Gosling, & Charalambous 2002). The rate of re-emergence of tuberculosis is an indication of the sterilising activity (Burman 1997).

Streptomycin was first used as a single drug against tuberculous meningitis in 1946. Tuberculous meningitis was at that time invariably fatal. A good response was recorded for 12% of children under 3 years and 36% of older patients. This trial on miliary tuberculosis produced little drug resistance (Medical Research Council 1948).

Streptomycin trials on pulmonary tuberculosis appeared initially as favourable as those on tuberculous meningitis. Improvements in both bacteriological and radiographic measures were apparent. However, drug resistance emerged in 35 of 41 (85 %) of patients and mortality after 5 years was only slightly improved in streptomycin treated patients (Fox, Sutherland & Daniels 1954). There was almost universal selection of streptomycin resistance in pulmonary tuberculosis, whilst tuberculous meningitis did not select resistant strains. This difference was judged to be caused by the greater baccillary load in pulmonary tuberculosis and so the greater probability of a rare resistant mutant occurring.

In 1948 combination therapy using para-aminosalycilic acid (PAS) and streptomycin was used in a trial of 166 patients. The use of combined therapy resulted in reduced emergence of streptomycin resistance and the rate of streptomycin resistance occurred more frequently as PAS dosage was reduced (British Medical Council 1950; Fox & Sutherland 1956), indicating that PAS did inhibit the emergence of streptomycin resistance.

Drug resistance in *M. tuberculosis* arises from alteration of the genome with no transferable resistance genes. This occurs most commonly via single base mutation but insertion sequences can interrupt gene sequences and so produce resistance (Ramaswamy & Musser 1998; Lemaitre, Sougakoff, Truffot-Pernot et al. 1999). The use of combined therapy provided a defence against emergence of resistance, as mutants resistant to both drugs must occur on the same genome. If the probability of each resistant mutation being present is 10^{-6} (one in a million) then the probability of both arising simultaneously in the same genome is (10^{-6} x 10^{-6}) 10^{-12} (Shimao 1987).

Isoniazid became available in 1952. Treatment with isoniazid alone as with streptomycin rapidly produced strains resistant to isoniazid. Trials of combination therapy involving PAS, streptomycin and isoniazid were carried out. The use of streptomycin and isoniazid was found to almost completely suppress the emergence of drug resistance and had the highest bacteriological cure rates. The proportion of patients treated with isoniazid and streptomycin with negative cultures at 3 months was 67% of

117 compared to 55% of 83 streptomycin and PAS treated patients (Fox 1953; Medical Research Council 1953b; Medical Research Council 1953a).

The use of two antimycobacterial agents in an infection caused by a strain of tuberculosis resistant to one agent, results in only a single effective agent being used. Triple therapy involving streptomycin, isoniazid and PAS was initially used to prevent treatment with a single effective antibiotic against tuberculosis resistant to a single therapeutic agent. Triple therapy improved the success rate of therapy from an unfavourable response at one year of 16% to only 3% (Medical Research Council 1962). These trials also indicated that prolonged periods of treatment were required. Relapse occurred in 62% of patients treated for 6 months, 19% in those treated for one year and 4% for those treated for 2-3 years. A single agent could however be effectively used for the continuation phase.

Triple therapy trials indicated that intensive combination therapy was only necessary during the initial 2 months of treatment. If therapy was incomplete during this intense period, failure was accompanied by development of drug resistance (Mitchison 1998). Treatment failure in the continuation phase would also result in relapse, but the infecting strain would not gain additional antibiotic resistance. This emphasises the importance to the emergence of drug resistance of large bacilli numbers, as was identified earlier with the comparison of miliary and pulmonary single agent treatment.

Experimental infection of mice with *M. tuberculosis* revealed that isoniazid alone or in combination with streptomycin or (PAS) produced a 2-log drop in viable cultures in two months. Continued treatment was however essentially bacteriostatic with little or no further reduction in spleen culture. The addition of pyrazinamide produced sterile cultures after 4 months of treatment (Burman 1997). Testing against different phases of growth of *M. tuberculosis* has identified a decline in activity of isoniazid as Mycobacteria move into non-growing phases of growth (Yamori, Ichiyama, Shimokata et al. 1992).

The environment that the tubercle bacilli occupy has had a major effect on the selection of antibiotics used. Mitchison (1992) has argued that tubercle bacilli undergo four different growth phases, and that different antibiotics used in therapy affect these phases. The four phases are:

1. A population with continuous growth and replication (often found in large numbers inside cavities): isoniazid is most effective against this population in terms of killing power but rifampicin and streptomycin are also effective.

2. A population composed of dormant organisms, for which no drug appears to be effective.

3. A population of bacteria with slow growth and occupying an acid environment, possibly within the macrophage. This population is affected by pyrazinamide.

Treatment with pyrazinamide for the first 2 months greatly reduces the probability of a relapse occurring.

4. Dormant organisms with intermittent spurts of growth: rifampicin is most effective against this population.

The different phases of growth of Mycobacteria cause a problem with predicting the outcome of antimycobacterial treatment. Mitchison's four-compartment model has two broad phases of *M. tuberculosis* infection. Actively growing bacteria against which routine susceptibility testing is aimed, and slow to stationary growth under acidic conditions. An antimycobacterial agent that affects the active growing phase will quickly eliminate *M. tuberculosis* found in the sputum. For complete eradication of *M. tuberculosis*, a sterilising agent capable of affecting the slow to stationary growth phase must also be used.

Following the emergence of drug resistance, it is necessary to use drugs based on the specific susceptibility pattern of the infecting strain. This therapy must take into account not only the need to ensure against further drug resistance by use of multiple antibiotics, but the different environments that need to be treated. Therapy must not only kill the rapidly growing large cavities of tuberculosis leading to smear negativity with apparent cure, but sterilise the more slow growing or dormant organisms to prevent re-emergence of tuberculosis.

1.3 The current world wide spread of tuberculosis

A continuous decline in tuberculosis was disturbed in the 1980s, when for the first time in the developed world tuberculosis cases stopped declining. In the United Kingdom the lowest number of reported cases was in 1987. In the ten years between 1988 and 1998 the reported number of tuberculosis cases in England and Wales increased by 21%. This increase was predominantly in London where tuberculosis cases increased 71 % over this period (Rose, Watson, Graham et al. 2001)

There has been a similar rise in tuberculosis in the United States of America. The causes of this increase in both the USA and UK have been largely due to HIV and immigration. In the USA 30% of the increase in tuberculosis from 1984 to 1990 was due to HIV (Schurmann, Nightingale, Bergmann et al. 1997). In Western Europe tuberculosis is found largely in immigrant groups from areas where tuberculosis is endemic (EuroTB 2003).

Tuberculosis remains the most common infectious cause of death with an estimated 3 million deaths annually (World Health Organization 2003). The chronic nature of TB means that headline figures, such as death rates of 3 million world wide, must be tempered with the much larger number of reported cases.

The US government set up the Advisory Committee for the Elimination of Tuberculosis (ACET). This proposed a simple plan outlined in the Weekly Epidemiological Record (Anonymous 1989).

1. To concentrate on high risk groups.
2. To improve surveillance.
3. To improve case prevention, particularly through chemoprophylaxis.
4. To strengthen communication activities.

For the majority of countries, prevention through chemoprophylaxis for individual cases as in proposal 3 is likely to be less appropriate than in the USA. Emphasis instead is applied to effective case finding (Anonymous 1989).

The rise in tuberculosis cases world wide from 1985 to 1992 led the WHO in March 1993 to declare TB a 'global health emergency'. The first time that the WHO has ever so singled out any disease (Reichman 1996).

The WHO has set itself a global target of treatment of 70% of all TB cases and treatment success of 85%. This target was initially set to be achieved by the year 2000. This target has now been moved to the year 2005. In the WHO report 2002 a smear case detection rate of 37% was reported with 82% successfully treated (World Health Organization 2004) .

A key component of this control is DOTS (Directly Observed Therapy Short term). This strategy involves a standardised therapy regime with an observer ensuring compliance over the period of therapy.

Globally the incidence of TB is increasing at about 0.4% per year, with much higher growth rates being reported in the former Soviet Union (6% per year) and in Eastern and Southern African countries most affected by HIV (5% per year) (World Health Organization 2003).

The latest WHO data refers to the year 2002 at which point 69% of the world population was covered by DOTS services (World Health Organization 2004). In that year 3.0 million new cases of TB were reported to WHO of which 1.4 million were smear positive cases. The importance of adequate tuberculosis control programs is illustrated by the estimate that, globally, 20% of patients default or fail to respond to therapy but less than 2% have MDR-TB (Dye, Scheele, Dolin et al. 1999). It is those patients who default on therapy who are most likely to develop MDR-TB (Pablos-Mendez, Gowda & Frieden 2002).

Drug resistance may either arise from exogenous infection by a strain that is already drug resistant (primary drug resistance), or from mutation and selection of resident strains of tuberculosis (acquired drug resistance). Small, Shafer, Hopewell et al. (1993) found that in 17 patients who had developed MDR-TB, four patients had an MDR-TB strain with a restriction fragment length pattern (RFLP) significantly different from the

initial drug susceptible strain. This indicated cross infection between patients leading to the supplanting of a resident drug susceptible tuberculosis strain with a drug resistant strain. The selective pressure of antibiotic usage will assist the invasion of a drug resistant strain, although the original drug resistant case was not identified. Van Rie, Warren, Richardson et al. (1999) reported exogenous infection with MDR-TB resembling strain 'W' in 2 HIV negative patients as a part of an MDR-TB outbreak involving 16 patients in a noninstitutionalised community. This indicates that drug resistance in tuberculosis infection can arise during treatment not only by acquisition of resistance mutations, but exogenous infection by MDR-TB.

Exogenous reinfection could be a major part of the tuberculosis relapse after successful treatment. Bandra, Gori, Catozzi et al. (2001) in a 5 year study in Lombardy, Northern Italy, identified 32 patients who had a tuberculosis relapse with a period of over 6 months between infections. Five (16%) of these patients' isolates had a significantly different IS6110 RFLP pattern in the second infection, indicating exogenous reinfection and not relapse of infection with the original isolate. Two of the exogenous reinfections were with a MDR-TB strain, however two of the reinfections were with a susceptible strain and one case was in the first infection an MDR-TB strain that caused two episodes of infection before reinfection with a susceptible strain. This indicates that reinfection is not just an event caused by drug resistant strains of *M. tuberculosis* and is not always driven by drug resistance.

Despite the WHO control program, MDR-TB (defined as *M. tuberculosis* resistant to isoniazid and rifampicin) is a continuing problem. Pablos-Mendez, Raviglione, Laszlo et al. (1998) reported a median incidence of MDR-TB in a survey of 28 countries of 2.2%, with a maximum incidence of 22.1% isolated in Latvia. Pablos-Mendez et al. (1998) reported the incidence of MDR-TB in Africa as low. This was described, as most likely due to the unavailability of rifampicin.

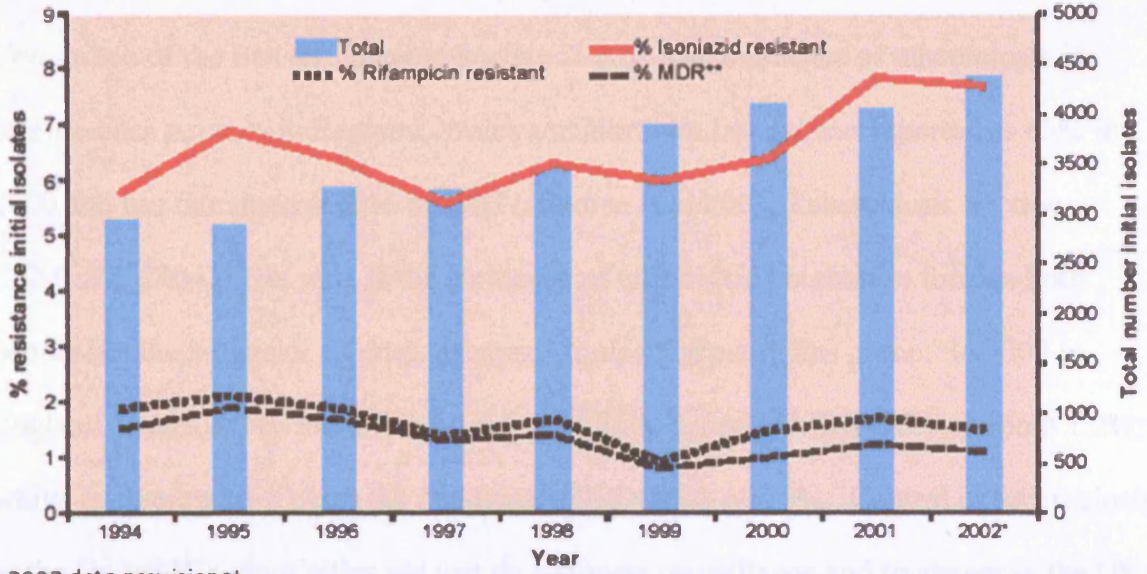
The announcement of the WHO control strategy has not, however, ended the problem of tuberculosis. Roselle, Danko, Kralovic et al. (2000) compared the US veterans' anti-tuberculosis hospital service to the US national service. Both systems used the DOTS strategy. The decline in reported tuberculosis in the veterans' service was significantly greater than that reported by Centre of Disease Control, Atlanta (CDC) for the national data. Tuberculosis declined in the veterans from 35.8 to 17.7 per 100,000. The CDC data declined over the same period from 10.4 to 7.4 per 100,000. The incidence of reported tuberculosis declined by nearly 50% in the Department of Veterans' Affairs, a large centrally administered health care system. The reported tuberculosis in the national data produced by CDC declined by 30%. The decline in reported tuberculosis in the veterans' service was obtained by extensive training of staff and infection control. This indicates again the importance of contact tracing and effective treatment.

In the UK a divided picture emerges with TB declining in rural areas but increasing in urban areas, particularly London. The overall rate of tuberculosis has been increasing since 1988 (see figure 1.1). In 2002 there were 6,974 cases of tuberculosis in England,

Wales and Northern Ireland (12.9/100,000 population). London contributed 3,005 (43%) of these cases (Tuberculosis Section 2004). The incidence of drug resistance however remains stable about 1.4% isoniazid resistant and 1.2% being multi-drug resistant tuberculosis (Antoine, Gatto & Story 2003).

The division between rural and urban rates of tuberculosis is not unique to the United Kingdom. Hayward, Darton, Van-Tam et al. (2003) in a survey of 20 cities in Western Europe came to the conclusion that tuberculosis in Western Europe 'is primarily a problem of large cities'.

Figure 1.1 Number of isolates in England and Wales and anti-TB drug resistance in initial isolates (graph taken from Tuberculosis Update 2004).



* 2002 data provisional
** Multiple Drug Resistant (at least isoniazid and rifampicin)

The figures for TB rates in Europe represent the difference between western and eastern nations especially the former Soviet Union nations. In surveillance of tuberculosis in Europe (2003) it was reported that in the west, a rate of approximately 12 cases per 100,000 is reported with declining notification rates in all but 4 nations. In the east, in the newly independent states of the former Soviet Union a rate of 90 per 100,000 population was reported. The figures for primary drug resistance again show a marked increase in the eastern (4-12%) as against western nations (0.7%) within Europe.

Maguire, Dale, McHugh et al. (2002) estimated a recent rate of transmission of tuberculosis of 14.4% from a molecular epidemiological study of tuberculosis in London. The majority of tuberculosis in London was either reactivation of infection or importation by recent immigration from endemic areas. In the control and prevention of tuberculosis in the United Kingdom 56% of TB cases reported in England, Wales and Northern Ireland during 1998 were in people not born in the UK (Joint Tuberculosis Committee of the British Thoracic Society 2000). The incidence of tuberculosis in foreign-born patients in England, Wales and Northern Ireland was reported as 63% in 2000 and has remained at 63% in 2002 (Antoine et al 2003, Tuberculosis Section C.D.C.S.C 2004). Not only is the incidence of tuberculosis highest in foreign-born people but the incidence of drug resistance is also highest in this group. In 2000 in England, Wales & Northern Ireland the overall incidence of MDR-TB was about 1.2%, whilst in foreign-born cases the incidence of MDR-TB was 8%. Control of tuberculosis in the United Kingdom relies not just on adequate surveillance and treatment in the UK but also in potential immigrants.

Tuberculosis has long been associated with poverty and overcrowding (Iseman 1994). This increase will occur predominantly in the less developed regions of the world (see table 1.1). The WHO has defined a mega city as a population of over 8 million. The numbers of mega cities are predicted to increase. The increasing concentration of people in mega cities, added to an increasing HIV incidence, produces an ideal environment for the transmission of tuberculosis. Rapid transport of people around the world means that tuberculosis from third world slums can spread into first world cities.

Table 1.1. World wide growth of mega cities (Horton 1996).
These are the 1996 estimates of where the number of mega cities would be formed and their geographical position

	1970	1994	2000	2015
Less developed regions				
Africa	0	2	2	3
Asia	2	10	12	19
Latin America	3	4	5	5
More developed regions				
Europe	2	2	2	2
Japan	2	2	2	2
North America	2	2	2	2
World	**11**	**22**	**25**	**33**

The WHO 1989 stated that tuberculosis world-wide occurs mostly from reactivated disease (Anonymous 1989). HIV both predisposes the infected person to tuberculosis and raises the probability that the disease will re-emerge (Schurmann et al 1997). The lifetime probability of an immunocompetent-infected person developing clinical

tuberculosis has been estimated as 10%. The annual probability of a patient coinfected with *M. tuberculosis* and HIV has been also estimated at 10%. HIV disease is predicted to increase, particularly in the less developed world, where the bulk of *M. tuberculosis* infected people live (Anonymous 1996). The increased likelihood for re-emergence of tuberculosis in this group is likely to lead to an increasing incidence of tuberculosis.

The increased susceptibility of HIV infected patients to tuberculosis can not be ignored as a factor in the spread of tuberculosis. Schürmann et al. (1997) estimated that in the USA, 40% of HIV positive tuberculosis patients were due to primary, not reactivated, disease. In the UK, tuberculosis and HIV co-infection has been estimated to contribute 8.5% of the increase in tuberculosis notification between 1993 to 1998 (Joint Tuberculosis Committee of the British Thoracic Society 2000). Increasing numbers of highly susceptible individuals have lead to an increase in tuberculosis. This has occurred in the USA where the incidence of *M. tuberculosis* infected healthy individuals is low and so the probability of an HIV infected host being infected with tuberculosis is low.

Schurmann et al (1997) outlined factors associated with the spread of tuberculosis. Amongst these factors were failure to take an adequate number of anti-tuberculosis drugs for a sufficiently long time and failure to modify drug regimens based on sensitivity test results. Both of these failures can lead to increasing drug resistance in *M. tuberculosis.*

Treatment of HIV sero-negative patients infected with MDR-TB, with treatments based on susceptibility data, can lead to good responses both clinically and microbiologically (Telzak, Sepkowitz, Alpert et al. 1995). Iseman (1993) in a review of MDR-TB infections found that 55-88% of MDR-TB infections responded favourably to treatment, the risk of failure being 80 times higher than that of drug susceptible TB infections.

Eltringham & Drobniewski (1998) report that relapses are unacceptably high in HIV sero-positive patients co-infected with MDR-TB if less than 18-24 months of appropriate treatment is used beyond culture conversion. Treatment of patients who are both HIV sera positive and infected with MDR-TB, may need to be continued for life and the outcome of treatment is less favourable than for HIV sero-negative patients infected with MDR-TB.

1.4 Methods of susceptibility testing *M tuberculosis*

There are three basic methods of testing the susceptibility of *M tuberculosis* to antitubercular agents. These methods are: the resistance ratio, the absolute concentration technique and proportion technique (Drobniewski 2002).

The resistance ratio technique uses Löwenstein-Jensen (LJ) media (Drobniewski 2002). This is an egg based media requiring insipisation at 80°C to both sterilise the media and to solidify the egg protein to form a solid slope. Heat and high protein concentration will affect the concentration of free antibiotic, by heat degradation and protein binding

respectively. The effective concentration present in a batch of LJ media will vary between batches and antibiotic susceptibility testing must therefore determine the concentration of free active antibiotics as well as determine the susceptibility of the strain being tested. To control the media, a set of at least six known susceptible strains must be tested on each batch of media. The concentrations of streptomycin, isoniazid, rifampicin, and ethambutol routinely used in the resistance ratio technique are given in table 1.2a. The modal MIC of the known susceptible strain is then used as the criteria to judge susceptibility or resistance of the test strains. A test strain with an MIC of no more than double that of the modal MIC of the known susceptible strain is considered susceptible. A test strain with an MIC of greater than 4 times that of the test strains is resistant and a strain with an MIC of 8 times that of the test strains is highly resistant (Collins, Lyne & Grange 1989)

The absolute technique determines the MIC of a strain of tuberculosis growing on either Löwenstein Jensen media, Middlebrook agar or Middlebrook broth (Drobniewski 2002). The size of inocula needs be very carefully controlled to produce reproducible results.

The proportion technique uses Middlebrook agar or broth (Drobniewski 2002). The definition of resistance is growth of more than one percent of the inoculum. This definition of resistance controls for variation in the inoculum size. This technique has been reliably used in break point style susceptibility tests. In break point susceptibility tests an MIC of above a specified concentration is defined as resistant. Break point susceptibility tests measure the isolate's ability to grow at a single drug concentration

(the concentration defined as resistant) (Hamilton-Miller 1997). The recommended concentrations of streptomycin, isoniazid, rifampicin and ethambutol used in Middlebrook 7H10 agar are given in table 1.2b. This method of testing susceptibility using a single antibiotic concentration has lent itself to mechanisation and has been used as the basis for a number of rapid susceptibility tests (Scarparo, Ricordi, Ruggiero et al. 2004; Marttila, Marjamaki, Viljanen et al. 2003).

The resistance ratio uses as a definition of susceptibility, the concentration of antibiotic that is capable of inhibiting the growth of the control strains. This is a population-based definition of susceptibility. This differs from the absolute and proportional techniques, which defines susceptibility as inhibition being exerted by a specified concentration of antibiotic. In practice these definitions may be very similar, and comparisons of susceptibility by proportional and resistant ratio techniques produce comparable results on clinical isolates, if differing MIC values (Laszlo, Gill, Handzel et al. 1983).

The minimal inhibitory concentrations of antibiotic in egg based media such as Löwenstein Jensen media can be higher than the MIC determined in more defined media such as the Middlebrook media (see tables 1.2a and 1.2b). Rastogi, Goh & David (1989) using the proportional technique on Middlebrook 7H10 agar and Löwenstein Jensen media used a concentration of 40 μg/ml rifampicin in Löwenstein Jensen media and 1μg/ml rifampicin in 7H10 agar. The concentration of ethambutol (2 μg/ml) and isoniazid 0.2 μg/ml were, however, the same in both media. As the antibiotic

concentrations used in screening for resistance vary with the media used, published MIC figures in the literature using different media may not always be comparable.

A problem with *in vitro* susceptibility testing is that it tests a single criterion: the ability of a drug to inhibit the growth of actively growing bacteria. This ignores the need to affect the differing populations of *M. tuberculosis* described by Mitchison (1992). Antitubercular susceptibility tests are normally only targeted at the population of mycobacteria actively growing in a normal pH. The susceptibility testing of pyrazinamide is the obvious exception. Pyrazinamide is active only at an acid pH so susceptibility testing must take place in an acid environment.

There is a need to develop therapy against the full range of environments described by Mitchison (1992) and their accompanying infecting populations. This has led to the need to test new antitubercular agents against *M. tuberculosis* both at normal pH and acid pH (Salfinger & Heifets 1988; Gomes, Paul, Moreira et al. 1999), as well as testing the anti-tubercular agent against *M. tuberculosis* in rapid and slow growth phases (Yamori et al. 1992).

Table 1.2a Drug concentration used to test for drug susceptibility used in resistance ratio technique using Löwenstein Jensen medium (Collins et al 1989).

	Final concentration (mg/L)						
Isoniazid	0.007	0.015	0.03	0.06	0.125	0.25	0.5
Ethambutol	0.07	0.15	0.31	0.62	1.25	2.5	5.0
Rifampicin	0.53	1.06	3.12	6.25	12.5	25	50
Streptomycin	0.53	1.06	3.12	6.25	12.5	25	50

Table 1.2b Drug concentration used to define resistance using proportion technique on solid media. Resistance being defined as growth of over 1% of inocula (Pablos-Mendez et al. 1998).

Drug	Concentration tested (mg/L)
Isoniazid	0.2
Ethambutol	2
Rifampicin	1
Streptomycin	4

The effect of the antibiotic does not disappear as the antibiotic falls beneath the MIC. Post antibiotic effect can have a major effect on dosage timing as can sub inhibitory concentrations. Short pulses of antitubercular drugs can inhibit the growth of *M. tuberculosis* for two to seven days (Burman 1997).

Wallis, Patil, Cheon et al. (1999) have described drug tolerance in *M. tuberculosis* to antitubercular agents. The MIC does not differ from drug susceptible strains of *M. tuberculosis* but the killing rate of isoniazid and rifampicin is reduced in tolerant organisms. Wallis et al. (1999) identified drug tolerance in two strains of *M. tuberculosis* where infection relapsed after successful treatment. The time taken for an infection to clear for rapid growth positivity, defined as growth in Bactec 460 media within 20 days of inoculation from a sample, has been correlated to the relapse of tuberculosis infection. A direct correlation between the time to clear for rapid positivity and drug tolerance of the bacilli was identified. Wallis et al. (1999) suggest that drug tolerance may be related to the ability of the bacilli to reside in the stationary phase. This would emphasise the importance of population two in Mitchison's model: dormant organisms at normal pH against which no antibiotic appears active.

The use of molecular probes to detect resistance opens the opportunity for rapid detection of antibiotic resistance (within 24 hours) without the need to isolate the organism. The low incidence of rifampicin mono resistance (0.2%) (Pablos-Mendez et al. 1998) makes probes for resistance to this antibiotic particularly valuable, detection of

rifampicin resistance very frequently means the strain is multi-drug resistant. A commercial probe (Rossau, Traore, De Beenhouwer et al. 1997) has been developed to both identify the presence of *M. tuberculosis* and detect rifampicin resistance directly from samples, enabling both identification of *M. tuberculosis* and detection of rifampicin resistance within 24 hours of receipt of samples by the laboratory. However, molecular techniques can only identify resistance mutations identified from association with high MIC values determined using growth based sensitivity tests.

1.5 Activity of rifampicin and development of resistance to rifampicin

Rifampicin is in the rifamycin group of antibiotics. This group is based on the fermentation products of *Amycolaptopsis mediteranei* (Parenti & Lancini 1997).

The different antibiotics in the rifamycin group have the same activity, inhibiting the elongation step of RNA polymerase. Rifampicin binds to free RNA-polymerase and blocks the formation of a stable DNA-RNA-polymerase complex. This blockage leads to an accumulation of aborted RNA products (Wehrli 1983). The different side groups on the rifamycins affect the permeability of the antibiotic across the cell wall affecting the MIC and pharmacological properties (Parenti & Lancini 1997).

Resistance to rifampicin can arise either from mutation of RNA-polymerase blocking the binding of rifampicin to RNA-polymerase, or altered cell wall permeability inhibiting

access of rifampicin to RNA-polymerase (Ramaswamy & Musser 1998; Hui, Gordon & Kajioka 1977).

The prokaryote RNA-polymerase core enzyme consists of four proteins, 2α chains, a β chain and β' chain. The binding of a sigma protein to the core enzyme creates the holoenzyme. The core enzyme can produce RNA from single stranded or nicked DNA, but is not able to bind to double stranded DNA or initiate strand separation. The holoenzyme is capable of recognising RNA promoter sites, separating DNA strands and initiating specific RNA synthesis. This indicates the sigma protein is responsible for RNA synthesis initiation. The sigma protein after initiation disassociates from the enzyme and the core enzyme continues elongation and termination of the RNA product. The transcription termination step can either be intrinsic, so that no other proteins are involved and signals beyond the termination sequence are not required, or a rho-dependant system exists. The rho-dependant system of transcription termination requires protein factors interacting with RNA-polymerase to effect termination (Von Hippel 1998).

Binding different sigma proteins to the core enzyme allows different initiation sites to be recognised and so enable different RNA transcripts to be produced in response to different environmental stimuli.

Heil & Zillig (1970) recombined RNA-polymerase from rifampicin sensitive and resistant bacilli. Resistance to rifampicin only arose where the β' polypeptide from the resistant clone was present. The β' polypeptide is coded for on the *rpo*B gene.

The *rpo*B gene of *E. coli* can mutate in three regions to confer rifampicin resistance. Telenti, Imboden, Marchesi et al. (1993a) used these regions as the basis for a search for rifampicin resistance in *M. tuberculosis*. Telenti's study found a single region of the *rpo*B gene involved in rifampicin resistance in *M. tuberculosis.*

Permeability of the cell affects rifampicin resistance. A number of studies have compared the MIC of various *M. tuberculosis* isolates with rifampicin resistant mutations to different members of the rifamycin group (Moghazeh, Pan, Arain et al. 1996; Bodmer, Zurcher, Imboden et al. 1995; Ohno, Koga, Kohno et al. 1996). These studies indicate that for all the rifampicin resistant mutations in the *rpo*B gene the MIC increases for all drugs of that class. However, for some *rpo*B mutations clinical susceptibility is retained for rifamycins with greater ability to cross the mycobacterial cell wall.

Wehrli (1983) purified RNA-polymerase from rifampicin resistant coliforms. The concentration of rifampicin required to inhibit by 50% the rate of RNA production, the ED_{50}, of the purified RNA polymerase was then determined. Wehrli found there was no direct link between the MIC and the ED_{50} of these resistant coliforms. This indicates

that the MIC is affected both by cell wall permeability and the binding affinity of rifampicin to RNA-polymerase.

1.6 Development of probes for mutation in the *rpo*B gene

Telenti, Imboden, Marchesi et al.(1993b) cloned the *rpo*B gene of *M. tuberculosis*. A PCR was designed based on this sequence to amplify a 411 bp fragment of the *rpo*B gene from rifampicin resistant clinical isolates of *M. tuberculosis*. Single stranded conformation polymorphism (SSCP) technique was used to identify the presence of mutations in the PCR fragments from the *rpo*B gene.

SSCP identifies mutations by separation of single stranded DNA (ssDNA) on non-denaturing gel using electrophoresis (Hayashi 1991). Single stranded DNA under non-denaturing conditions refolds as a result of self-complementary and intramolecular interactions. This folding is dependent on the sequence and results in a unique three-dimensional shape for the DNA strand. Alteration of a single base per 400 base pairs alters the intramolecular interactions resulting in an altered shape.

Mutation will not always produce a recognisable change in SSCP patterns under all conditions (Vidal-Puig & Moller 1994). The presence or absence of glycerol (Vidal-Puig & Moller 1994) and temperature (Hongyo, Buzard, Calvert et al. 1993) of the gel can affect the sensitivity of the technique. However this technique has been used successfully by myself for screening mutations both in the *rpo*B gene (Billington,

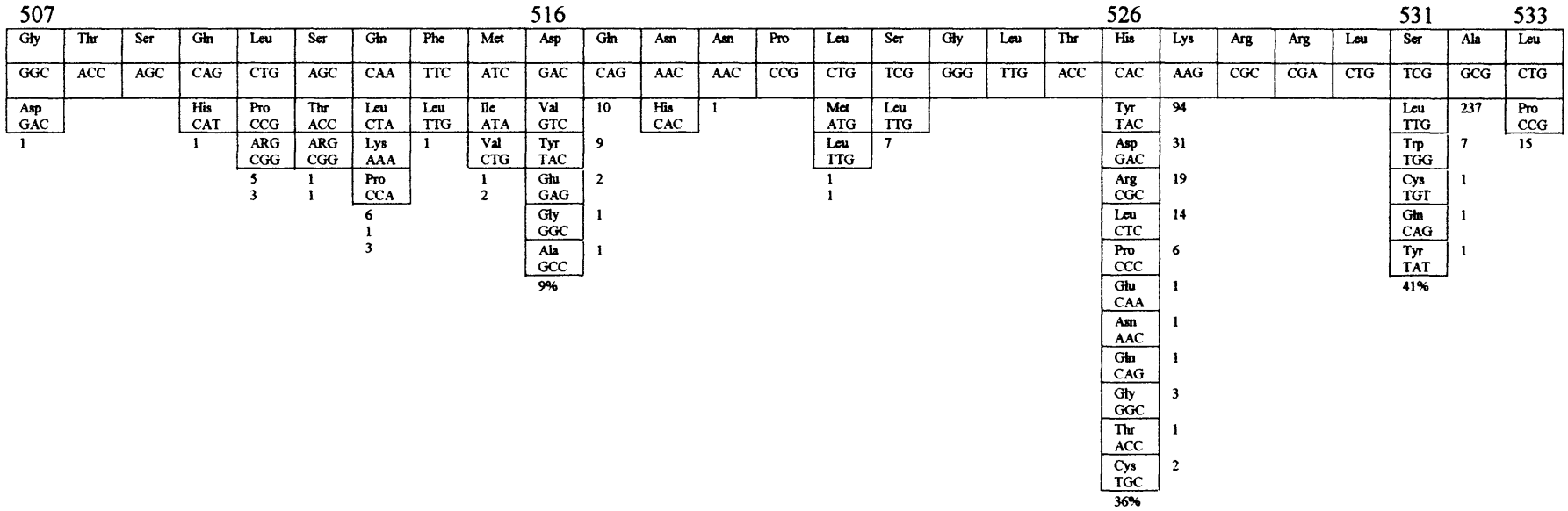
McHugh & Gillespie 1999) and the PZA gene (Hannon, McHugh, Billington et al. 1997; Davies, Billington, McHugh et al. 2000b) in *M. tuberculosis*.

The mutations in the *rpo*B gene of *M. tuberculosis* identified by Telenti et al. (1993a), were clustered in a single region coding for 23 amino acids (69bp). Telenti et al. (1993b) using PCR-SSCP were able to identify mutations present in clinical isolates from the band position on PCR-SSCP which were confirmed by sequencing.

The mutations identified by Telenti et al. (1993b) and Telenti et al. (1993a) were single base substitutions. A point mutation is defined where a single base is replaced by one of the other three possible bases (Drake 1991a). Ramaswamy & Musser (1998), in a review of the literature on molecular methods of resistance in *M. tuberculosis*, reported that by extending the region of the *rpo*B gene amplified by PCR to an 81bp core (coding for 27 amino acids) 96% of rifampicin resistant *M. tuberculosis* strains can be detected. Ramaswamy & Musser (1998) confirmed that the majority of rifampicin resistant mutations in the core region of the *rpo*B gene are single base substitutions (see figure 1.2 for base substitutions identified in clinical isolates).

Ramaswamy & Musser (1998) identified a small proportion (4%) of clinical isolates with no apparent mutation in the *rpo*B gene. These rifampicin resistant isolates do not appear to have any mutations in the *rpo*B gene and the mechanism of resistance is unknown. Heep, Rieger, Beck et al. (2000) have identified rifamycin resistant mutations outside of the region on the *rpo*B gene identified by Telenti et al. (1993a)

Figure 1.2 Mutations located in positions 507 through to position 533 of the *rpo*B gene of *M. tuberculosis* taken from Ramaswamy & Musser (1998). 478 rifampicin resistant mutations were summarised in this diagram. The numbers correspond to the *E. coli* RNA-polymerase amino acid positions. The figures give the number of reported isolates. Only those mutations arising from base substitution (point mutation) are given in the diagram. The percentage of rifampicin resistant mutants are noted for positions 516, 526 and 531, these three positions accounting for over 80% of all rifampicin resistant isolates. 38 individual substitutions lead to rifampicin resistance. Of these 31 are single base substitutions (single point mutations) and 7 involve 2 base changes (two point mutations).



1.7 The development of resistance in the host

The use of triple therapy involving isoniazid, rifampicin and pyrazinamide does result in occasional failure and development of antibiotic resistance. This development of resistance has been associated with poor compliance by the patient (Mitchison 1998). A patient neglecting to take all of the antibiotics prescribed is effectively subject to monotherapy. The unsuccessful trials of monotherapy against pulmonary tuberculosis indicated the necessity for combined therapy. The use of a number of antibiotics ensured that clones of bacilli resistant to one antibiotic are affected by other drugs being used. The development of DOTS was to prevent poor compliance, each patient being directly observed to take the full drug regimen.

Antibiotics have different absorption rates across the gut and into various tissues. Antibiotics are similarly excreted at different rates. Pharmocokinetic data such as levels of antimicrobial agent present can, with varying degrees of difficulty, be determined. Serum levels of antimicrobial agents are relatively easily obtained and tissue levels can then be determined. The macrophage is the normal site of infection for *M. tuberculosis* and penetration into this site of anti-tubercular agents has been determined (see table 1.3). Other symptoms and concurrent diseases of the patient affect the drug absorption and excretion rates. Antibiotics can rise to different peaks within different regions of the body due to the difference in excretion and absorption rates in a similar fashion to the way that chemicals are separated by chromatography in the laboratory. This process leads to bacteria being exposed to periods of, what is effectively monotherapy..

The extended pharmacological half-life of rifapentine can result in this antibiotic being present after other antibiotics simultaneously administered have been excreted. The use of rifapentine, a rifamycin in the same antibiotic group as rifampicin, has become associated with the rare emergence of mono-rifampicin resistance (Vernon, Burman, Benator et al. 1999).

This model of pharmacological compartments, each with different antibiotic absorption and excretion rates, leading to periods of monotherapy, has been used as the basis for a model of antibiotic resistance development. Multidrug resistant strains accumulate each resistance sequentially.

Mitchison (1998) describes a ratchet mechanism based on these pharmacological compartments. Combined therapy is begun with all antibiotics initially present. Each antibiotic is then removed at different rates. This can produce a period of effective monotherapy during which a clone of monoresistant bacilli can expand. This clone is culled during effective therapy but then re-expands until such time as a multi drug resistant clone develops. This mechanism has been described in mathematical models (Lipsitch & Levin 1998).

Drug	Isoniazid	Streptomycin	Pyrazinamide	Rifampicin	Ethambutol
Minimum inhibitory concentration (MIC)	<0.05	<2.0	15	<0.25	<3.8
Minimum bactericidal concentration (MBC)	<0.05	8	50	<0.5	30
Maximum serum concentration (Cmax)	3-5	25-50	45	4-16	5-5
Serum half life (Hours)	1-4	2-3	8-10	2.5-5.0	3.5-5.0
Cmax/MIC	60-100	15-25	3	8-64	0.5-1.3
Intracellular concentration /Extracellular concentration	0.62-0.98	3.0	1.2	5.0	9.5
MIC for extracellular bacilli	<0.05	<2.0	15	0.02-0.12	1.0
MIC for intracellular bacilli	<0.05	5.0	Not consistently active	0.1-0.25	20
Intracellular MIC/Extracellular MIC	1.0	2.5	Not applicable	2.0-4.0	20

Table 1.3 Pharmokinetic characteristics of antitubercular drugs. All concentrations in μg/Litre (Burman 1997).

Baquero & Negri (1997) uses this compartment model to describe a mechanism in which extended β-lactamase developed in coliforms. Extended β-lactamase is capable of degrading third generation cephalosporins such as cefotaxime. The initial wild type β-lactamase TEM-1 lacks extended β-lactamase activity. TEM-10 differs from TEM 1 by two mutations and TEM 12 differs by a single mutation (see table 1.4).

Table 1.4 MIC determined in an *E. coli* strain given site directed mutagenesis to construct TEM-10 and TEM 12 from TEM-1 (Baquero & Negri 1997)

β-lactamase	mutation	MIC cefotaxime (μg/ml)
TEM-1	none	0.03
TEM-12	Arg164Ser	0.06
TEM-10	Arg164Ser & Glu240Lys	1.0

Baquero & Negri (1997) identified the MIC of *E. coli* containing β-lactamase containing the mutations identified in TEM-12 and TEM 10 created by site directed mutagenesis. TEM-12, with a single mutation at position 164, has only a slightly raised MIC. Mixed cultures of *E. coli* containing TEM-1 and TEM-12 subject to 4 hours exposure of cefotaxime allowed selective multiplication of TEM-12 when the concentration of cefotaxime was between 0.008 to 0.06 μg/ml. TEM-10 was selected from mixed cultures of *E. coli* containing β-lactamase TEM 1 and TEM 12 when exposed for 4 hours to a concentration of above 0.12 ug/ml. This led Baquero & Negri (1997) to the suggestion that extended β-lactamase is produced by sequential mutation. TEM-12 containing a single mutation is primarily amplified at low concentrations of cefotaxime.

TEM 12 then gains the second mutation leading to TEM-10 enabling growth in high concentrations of cefotaxime.

This model of sequential single antibiotic resistance raises the question as to whether the mutation rate for resistance can be determined, and what is the probability of resistance being present in a population.

1.8 Models of fitness and the movement of the genome in gene space

The relative fitness of a mutation, defined as the ratio of generations of the mutant and parental strain, can be determined. This led Wright (1932) to propose a theoretical fitness landscape. On this landscape all possible mutants and their respective relative fitness values can be plotted as a topographical map. This can lead to different landscapes. A single peak indicates a single optimum sequence. Multiple peaks or ridges on a fitness landscape indicate a number of genome sequences with equivalent relative fitness values.

Wright considered the evolution of a population of haploid organisms on a single peak fitness landscape. Wright described two types of genetic movement: selection and genetic drift. Selection leads the genome to climb the peak and achieve a population with a higher fitness than the parental strain (see figure 1.3). Genetic drift is the random movement of the genome across the fitness landscape, as more of the landscape has a

lower fitness it is into this region of the fitness landscape the genome is most likely to travel.

The definition Wright used for fitness was the relative number of progeny produced by the mutant and wild type. This measure can be produced both in the laboratory and can be measured in the natural environment. The advantage of laboratory studies is that defined bacteria differing by a single allele can be tested within a single constant environment (Lenski & Travisano 1994). The advantage of using fitness values from naturally occurring populations is that all of the environmental factors affecting that genome are present. The disadvantage is that the alleles present in naturally occurring populations differ widely. This variation in fitness produced by other alleles can mask variation in fitness of the character tested for.

Populations that are very small are liable to be highly affected by chance mutations. This chance mutation occupies a disproportionately high proportion of small populations. Sampling error can then lead to this mutation becoming adopted as the mean cell type. Wright described small populations as drifting across the fitness landscape. The role of chance becomes of greater importance than selective pressure in small populations. Such a population moves at random over the landscape, it is more likely to occupy one of the many positions with lower fitness on the landscape than the few positions with improved fitness (see figure 1.3).

The area of the fitness landscape occupied by the various genome sequences in the population is dependent on the mutation rate and the strength of selective forces. A high mutation rate or small difference in relative fitness between genome sequences will result in a larger area of the fitness landscape being occupied. This occupation of a large region of the fitness landscape indicates a high amount of genetic variation in the population. The amount of genetic variation is important in a changing environment. Selection acts where there exists in the population alleles better adapted to the environment than the population average. The higher the amount of genetic variation, the more likely it is that these 'better adapted alleles' exist. An optimum allele that is absent from the population can have no effect on that population (Bell 1997).

Eigen (1992) and Kauffman (1991) considered the size of gene space. Gene space is a mathematical model devised by Hamming (1980) linking the number of possible gene sequences of the same size with the number of steps or mutations required for one gene sequence to alter to a second. The size of gene space separated by a single substitution mutation is 3N, where N is the number of bases in the genome or gene considered. The size of gene space with two step mutations is $3N^2$ and where there are n step mutations is $3N^n$. The size of gene space increases logarithmically with increasing numbers of individual, or step mutations.

Eigen (1992) and Kaufman (1991) described gene space as a latticework of mutations. Each step on the lattice moved under selection must be such that the relative fitness

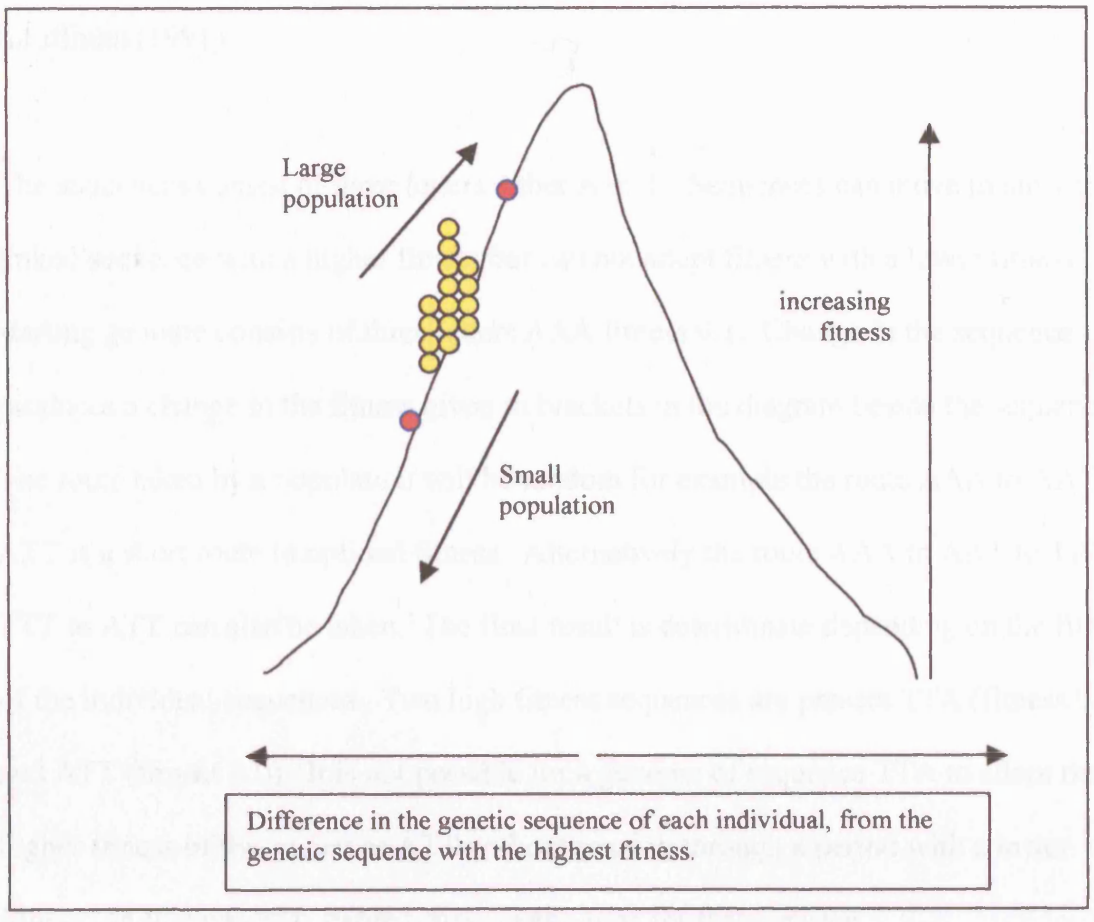
increases. This lattice network of gene-space can lead to a number of peaks, if two equally optimal choices leads to opposing regions of the lattice (see figure1.4).

The assumption is that a simple hill-climbing algorithm such as described by Mitchell (1996) operates (figure 1.5). Large populations can behave as predicted by this hill-climbing algorithm. Lenski & Travisno (1994) working with *E. coli* and Novella et al. (1995) working with vesicular stomatitis virus have identified an increase in fitness with culture and passage of large numbers of organism.

The passage of small populations of bacteria and virus are more likely to be affected by genetic drift. The few surviving reproductive organisms are subject to sampling error. There is a high probability that disproportionately high proportions of the passaged organisms are of an unusual genotype. The likelihood of drift resulting in reduced fitness is then proportional to the proportion of the culture occupied by unfavourable mutants. This drift to unfavourable or rare mutants present in the initial inocula has been referred to as the 'founder effect' (Bell 1997).

The repeated passage of bacilli or virus using small inocula and culture size will generate few mutations. The number of favourable mutants leading to increased fitness is far fewer than that of unfavourable mutants leading to a decline in fitness as measured by the ratio of generations of parent and mutant. This technique is likely to lead to repeated adoption of unfavourable mutants leading to a continued decline in fitness. This has been referred to as 'Muller's ratchet' (Muller 1964).

Figure 1.3 Population movement on a fitness landscape.



Genome sequences on a fitness landscape.

1. Each yellow dot represents an individual genome of the same population average fitness where there are n genomes. The two red dots are mutants of higher and lower fitness.
2. The larger the mutation rate or larger the population the more of the fitness landscape is covered by the population. This arises as there are more mutants present.
3. Each genome has a 1/n chance of being selected and dying regardless of position on the fitness landscape
4. The higher the position on the fitness landscape the more progeny are produced.
5. The movement of a large population is up the fitness landscape. Fitter mutants produce more progeny and so will if they survive become the population average. The fitter mutant may be selected and die but will be continually produced.
6. The movement of a small population is down the fitness landscape as more less fit mutants exist than more fit mutants. And the higher the probability that by chance the more fit population will be eliminated.

Figure 1.4 The latticework models of gene space proposed by Eigen (1992) and Kauffman (1991).

The sequences consist of three letters either A or T. Sequences can move to any other linked sequence with a higher fitness but can not adopt fitness with a lower fitness. The starting genome consists of three letters AAA fitness 0.1. Change in the sequence produces a change in the fitness given in brackets in the diagram beside the sequence. The route taken by a population will be random for example the route AAA to AAT to ATT is a short route to optimal fitness. Alternatively the route AAA to AAT to TAT to TTT to ATT can also be taken. The final result is determinate depending on the fitness of the individual sequences. Two high fitness sequences are present TTA (fitness 0.8) and ATT (fitness 1.0). It is not possible for a genome of sequence TTA to adopt the higher fitness of the sequence ATT without passing through a period with a lower fitness. In this way two stable fitness peaks arise for that genome.

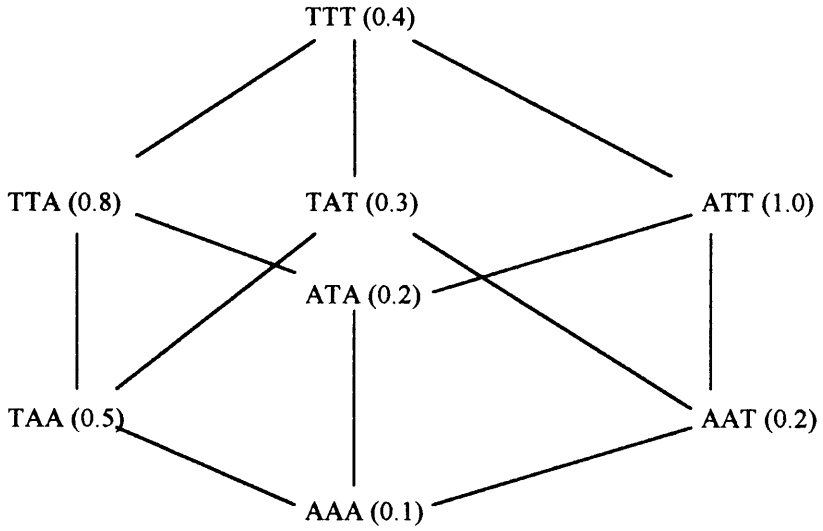


Figure 1.5 A hill-climbing algorithm.

The result of the algorithm is that a population of a higher fitness will be produced. This can be illustrated by assuming a population of organisms with a genome of 6 digits consisting of either 1 or 0. The fitness being the total number of 1s in the genome. The higher the number of 1s the more likely the genome is to be selected. Any mutation that converts a 0 to 1 will increase the fitness of that organism and so increase the likelihood of that genome being selected in future.

****

1. Start with a randomly generated population
2. Calculate the fitness of each member of the population
   a) Repeat the steps three to five until *n* offspring have been selected.
3. Select a member of the population at random, the probability of being selected being an increasing function of fitness. Selection is done 'with replacement' meaning the same member of the population can be selected more than once.
4. Mutate the offspring at each locus with probability *p*m (the mutation rate) and place any new mutants into the population.
5. Once *n* new members of the population have been selected replace the current population with the freshly selected population.
3. Go to step 2.

****

Clarke, Duarte, Moya et al. (1993) working with vesicular stomatitis virus repeatedly cultured individual plaques of virus. These plaques were derived from individual virus and by passaging these plaques Clarke et al (1993) restricted the culture at passage to an individual virus. These cultures revealed a continued decline in fitness as measured by the ratio of generations formed in competition with the parental strain of virus. This is an example of Muller's ratchet in the laboratory. Andersson & Hughes (1996) repeated this methodology using *Salmonella typhimurium* to reveal the same decline in fitness attributed to Muller's ratchet in bacilli.

Moran (1996) identified a more rapid rate of evolution in the aphid endosymbiot (genus *Buchnera). Buchnera* live in the aphid cell cytoplasm and are transmitted only to the progeny of the infected aphid. This introduces the low culture numbers and low passage number under which Muller's ratchet can operate. The high rate of genetic drift does not however indicate that *Buchnera* are poorly adapted to the aphid. *Wolbachia pipientis*, a member of the *Buchnera* genus, can determine the sex of progeny of the infected aphid host (Vines 1999). *W. pipientis* infecting the host cytoplasm are not present in spermatozoa, and as such are only transmitted by the female aphid. *W.pipientis*, by ensuring the infected aphid only produces female progeny, is able to ensure the maximum numbers of infected progeny are produced.

The accelerated evolution, or genetic change, introduced by Muller's ratchet need not be detrimental to virulence of the infecting bacilli as in the case of *W. pipientis*. Individual clones may decline in fitness due to poor adaptation to the host but competition then

continues between infected hosts introducing a larger population size. In this larger population the passage number is the number of infected aphids produced at each aphid generation.

M. tuberculosis is passed between patients by aerosol. This indicates a low infective dose. Such low inocula lead to the environment in which Muller's ratchet can operate. However, the amount of genetic variation between species in the *M. tuberculosis* complex is low. As stated earlier the five species making up the *M. tuberculosis* complex have a 99.9 % similarity at the nucleotide level (Kapur, Whittam & Musser 1994; Sreevatsan et al. 1997). This indicates that, unlike *W. pipientis*, there is not an accelerated rate of evolution.

1.9 The population size in tuberculosis infections

Tuberculosis infection undergoes a number of genetic restrictions. The infection is transmitted via an aerosol of droplet nuclei and a susceptible person can acquire infection by inhaling fewer than 10 bacilli (American Thoracic Society 2000; Barkley & Kupica 1994). A low infective dose indicates the infection starts with little genetic variation. This would enable the founder effect to have a strong influence on the continued tuberculosis infection. This would in turn favour genetic drift at the individual infected host level.

Frost & McLean (1994) studying the evolution of drug resistance in HIV found that the initial distribution of genetic variation had an important effect on the resulting emergence of drug resistance.

Tuberculosis can undergo prolonged periods of stasis. This stasis, it is assumed, occurs at the heart of foci of infection. These foci are created by delayed type hypersensitivity, which destroys infected host cells and surrounding material creating a solid centred cavity. These cavities with reduced blood flow leading to reduced oxygen supply, a reduced pH, and inhibitory fatty acids, present a harsh and unfavourable environment for *M. tuberculosis*, a strict aerobe (Dannenberg, Jr. 1992). For these foci to become the source for re-emergence of tuberculosis infection, bacilli must survive these conditions for decades. The numbers of bacilli involved are unknown but this could again present a genetic restriction. The evidence that single therapy in the continued phase of anti-tuberculosis therapy can eliminate this population indicates low bacilli numbers.

The development of drug resistance must involve a genetic restriction; the population is restricted to those cells containing mutations that are drug resistant. Tuberculosis infection not only undergoes severe genetic restriction but can undergo massive population expansion within the host. This can lead to an increase in genetic variation. This increase of the population and genetic variation could enable selective forces to operate within a single host, as well as genetic drift created by genetic restriction at transmission and selection of essential alleles.

The haploid reproductive system with no genetic transfer means that each genetic mutation produces in effect a novel lineage of bacilli. These lineages of bacilli then compete. The adaptation of *M. tuberculosis* can occur either from competition within the host generating fitter variants, which are then adopted within the host, or competition between hosts. Selection acts from competition between the frequency of infected individuals infected by differing lineages of bacilli.

The dogma of antibiotic control policies is that evolution of antibiotic resistance leads to a lower level of relative fitness (Levin, Lipsitch, Perrot et al. 1997). However it is possible that adaptation of *M. tuberculosis* to enhanced relative fitness would occur within the host. This would mean that a single MDR-TB case could lead to a lineage of bacilli as fit as the susceptible parental strain after passage within a single host. This would make a single MDR-TB case a possible epidemic.

The possibility of adaptation is dependent on the amount of variation prior to the exertion of lethal selective pressures such as antibiotic therapy. A low amount of variation will produce a high role for genetic drift. Allele selection for resistance will be dependent on the mutation rate. A large amount of genetic variation will lead to resistance selection being based more on the fitness of the allele.

1.10 Mutant frequency is dependent on when the mutation occurs

Luria & Delbrück (1943) proved that mutations occur independently to the application of a selective pressure in haploid populations. Luria & Delbrück postulated that a mutation occurring in a population occurs during cell division and leads to an altered phenotypic character. The mutant following its creation will divide and grow in the normal haploid fashion.

Luria & Delbrück predicted a single regular mutation rate, this will result in a Poisson distribution of mutation events, with the same probability of mutation at each cell division. The number of mutation events will be dependent on the population size (N) and the mutation rate (μ). The number of mutation events being on average $N\mu$.

The number of mutants will be dependent on when the mutation event occurred. A mutation that has only recently occurred will produce a single mutant cell. A mutant event occurring earlier in the broth will produce 2^g cells, where g is the number of generations since the mutation event.

The key prediction made by Luria & Delbrück (1943) was that the mutation frequency will have a high variation between experiments. The time of mutation creation has a greater influence on the frequency of mutants than the number of mutation events. A distinction must be made between the mutation rate and the frequency of mutant cells. The frequency of mutants is the number of mutant cells per cell in the total population;

this is more usually represented as the number of wild type per mutant. The mutation rate is the average rate at which mutation, the mis-incorporation or substitution of a base, occurs.

The mutant frequency is the product both of the mutation process, as well as the growth of the resultant mutants produced from mutation events. The frequency of mutant cells identified in a broth can vary widely. In figure 1.6 a pair of cultures are illustrated in which the total number of cells are identical. However in the first culture the mutation event occurs on the initial cell division after inoculation leading to half the cell culture being a part of that mutant lineage. In the second culture a single mutation event occurs in the last generation leading to a single mutant cell. Both cultures had a single mutation event, but the resulting frequency of mutants varies widely purely due to the timing of the mutation event.

The Luria-Delbrück distribution assumes that this mutation arises during replication of DNA and that the mutation event is as likely to affect any dividing cell. Jones (1994) illustrates the Luria-Delbrück distribution by imagining a synchronous dividing culture. In this culture one half of all the cells are produced at the last generation. This means as half of all the cells were produced in the last generation that one half of all mutation events are likely to have arisen in the last generation and one half of all mutation events will have produced a mutant cell lineage of one cell. One quarter of all the cells were produced in the generation before last so that in a synchronous culture one quarter of all mutation events are liable to have produced a mutant cell's lineage of two cells. In a

synchronous cell culture the probability of a mutation event at each preceding generation halves and the resulting number of mutant cells doubles. The probability of a mutation event producing a lineage of cells in this synchronous culture can be calculated as follows:

Equation 1.1 $Cr = 1/ 2r$

Where Cr is the probability of a mutation producing r cells,

where $r = 1,2,4,8,\ldots 2^n$.

Figure 1.6 Illustration of 2 cultures both with a single mutation event. In figure 1.6a the culture ends with one half of all cells being mutants. In figure 1.6b, the culture ends with a single mutant cell present.

Figure 1.6.a.

Schematic diagram of a culture in which a mutation event arises early in culture leading to half the cell culture being derived from a single mutation event (filled circles) and half being of parent type empty circles.

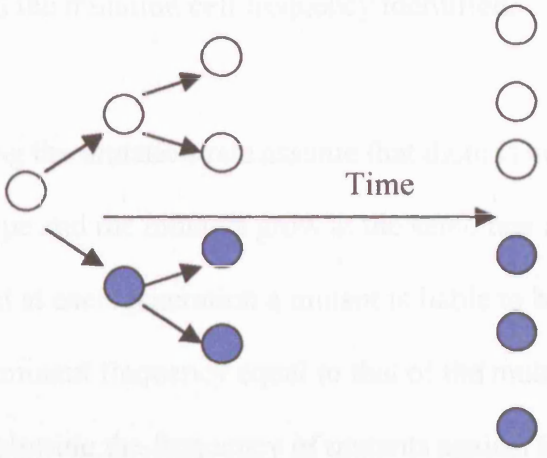
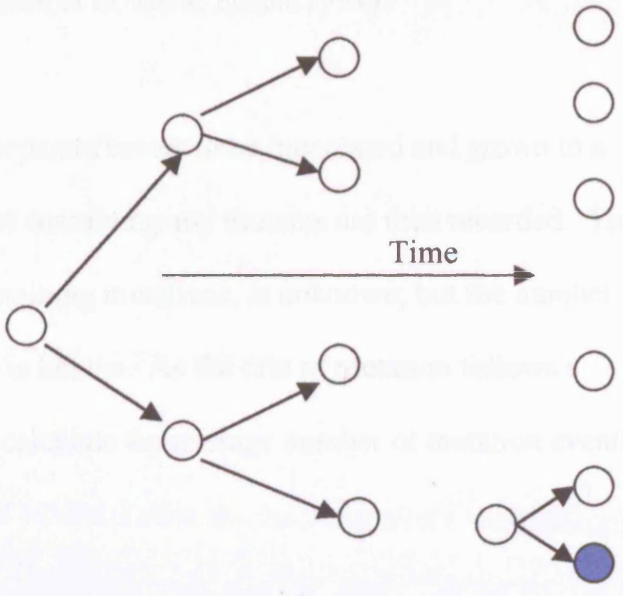


Figure 1.6.b.

Schematic diagram of a culture in which a mutation event arises late in culture leading to only a single mutant being present in the broth (filled circle).



1.11 Measurement of the mutation rate

Two strategies for determining the mutation rate have been devised. The first strategy is to attempt to determine the mutation rate directly from the Poisson distribution. The Po technique and continuous culture techniques use this approach. The second strategy is to use the Luria-Delbrück distribution to attempt to determine the most likely number of mutation events which will have produced the mutation cell frequency identified.

Continuous culture methods of determining the mutation rate assume that there is no back mutation rate from mutant to wild type and the mutants grow at the same rate as the wild type cell. In a large culture, such that at each generation a mutant is liable to be produced, there will be an increase in the mutant frequency equal to that of the mutation rate. The mutation rate is determined by plotting the frequency of mutants against the total number of cells in the broth over a period of time and then calculating the gradient of the resulting line (Bachl, Dessing, Olsson et al. 1999; Foster 1999).

The Po technique requires a number of separate broths to be inoculated and grown to a standard size. The numbers of broths not containing any mutants are then recorded. The number of mutation events in broths containing mutations, is unknown, but the number of mutation events in broths mutant free is known. As the rate of mutation follows a Poisson distribution, this can be used to calculate the average number of mutation events per broth (see equation 1.2).

Equation 1.2 $m=-\ln Po$

Where m is the average number of mutation events that have occurred in a series of broths and Po is the proportion of those broths in which no mutants were identified. The equation 1.2 assumes that all of the broth was sampled. Jones (1993) has calculated an equation for the Po technique taking into account only partial sampling of each broth.

The mutation rate is then calculated by dividing the average number of mutation events by the average cell numbers in each broth. This technique requires a simple scoring system: mutant cells being present or absent.

The frequency of mutants and the Luria & Delbrück distribution has been used as the basis of a number of methods for calculating the mutation rate. The use of the Luria Delbrück distribution to calculate the mutation rate requires that a series of broths need to be cultured so that mutants are present in all of the broths. The frequency of the mutant cells in these cultures is then determined. The mean number of mutants has been used to calculate the mutation rate. However, the effect of a single large mutant frequency can greatly affect the mean value and the median number of mutants is more accurate when used to calculate the mutation rate (Stewart 1994). Formulae for the calculation of mutation rate from the median number of mutants is given in section 2.11.

The most common lineage size or modal lineage size in the Luria-Delbrück distribution is one cell. The mean mutant lineage size is however greater than one. The normal and

Poisson distribution requires both the mean and median to be the same, where they differ there is a skewed distribution. Parametric statistical tests such as the t test based on the normal distribution should not be used to compare mutant frequencies. Non-parametric statistical tests such as the Mann-Whitney U test, which makes no assumption on the distribution, should be used where such skewed distributions are found.

1.12 The number of mutants in a large population

M. tuberculosis has no transferable mechanisms of resistance. Development of resistance is almost solely by chromosomal mutation and selection of those resistant mutations. Resistance due to insertion sequences disrupting the pyrazimidase gene to produce resistance have been reported (Lemaitre et al. 1999). Resistance by insertion sequence as with point mutations is not transferable between strains and so will act similarly to mutations but with different mutant frequencies. The mutations associated with the development of resistance to various antibiotics have been reviewed (Blanchard 1996; Ramaswamy & Musser 1998; Zhang & Young 1994). This raises the question: what is the probability of an antimycobacterial agent resistance mutant being present, in a population of *M. tuberculosis*, at the start of therapy?

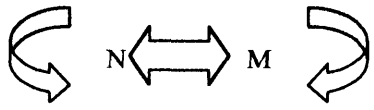
The number of bacilli in a mutant clone will be 2^g, where g is the number of generations since the original mutation event forming that clone. It is apparent that there will be more mutants as g increases. The more generations that have occurred in a culture, the higher the likelihood that a mutant clone exists in which g is greater than 1. This means the larger a culture, the more mutants are liable to be present and to have grown. In this

way the number of bacilli in culture will determine both the likelihood that a mutant is present and the number of mutants which are liable to be present.

A continuous culture in which a culture of constant cell frequency is maintained for a number of generations can undergo an infinite number of generations. The build up of mutant cell numbers will be dependant on the death rate of the mutant cell type in addition to the number of freshly created mutants and growth rate of the existing mutants. That is the fitness of the mutant defined as the ratio of generations of mutant formed per parent type cell generation.

Armitage (1952) outlined the relationship in the simple model outlined in figure1.7. The normal cell can mutate to form mutant cells, and the mutant cells can mutate back to form normal cells. Both mutant and normal cells replicate. In this way an equilibrium can be achieved between mutant and parent cell type.

Figure 1.7 The growth of mutants (M) and normal cell types (N).



The exclusion of the mutant cell type by competition with the fitter wild type cell formed in a suitably large culture does not mean the elimination of the mutant cell type. A mutant cell with zero growth rate and the same death rate as the normal 'parent cell type', can at each generation be formed afresh by mutation, if the parent cell culture is large enough. If one considers an idealised culture in which 10 new mutants are formed at each generation, these mutants are incapable of growth and die at a rate of 0.5 per generation. An equilibrium will be established in which the death rate is matched by the birth (mutation) rate (see figure 1.8). The mutant cell population will continue to expand until the number of dying cells exactly matches the fresh mutant numbers. That is 10 new fresh mutants formed from the parent cell population are matched by 10 deaths in the existing population.

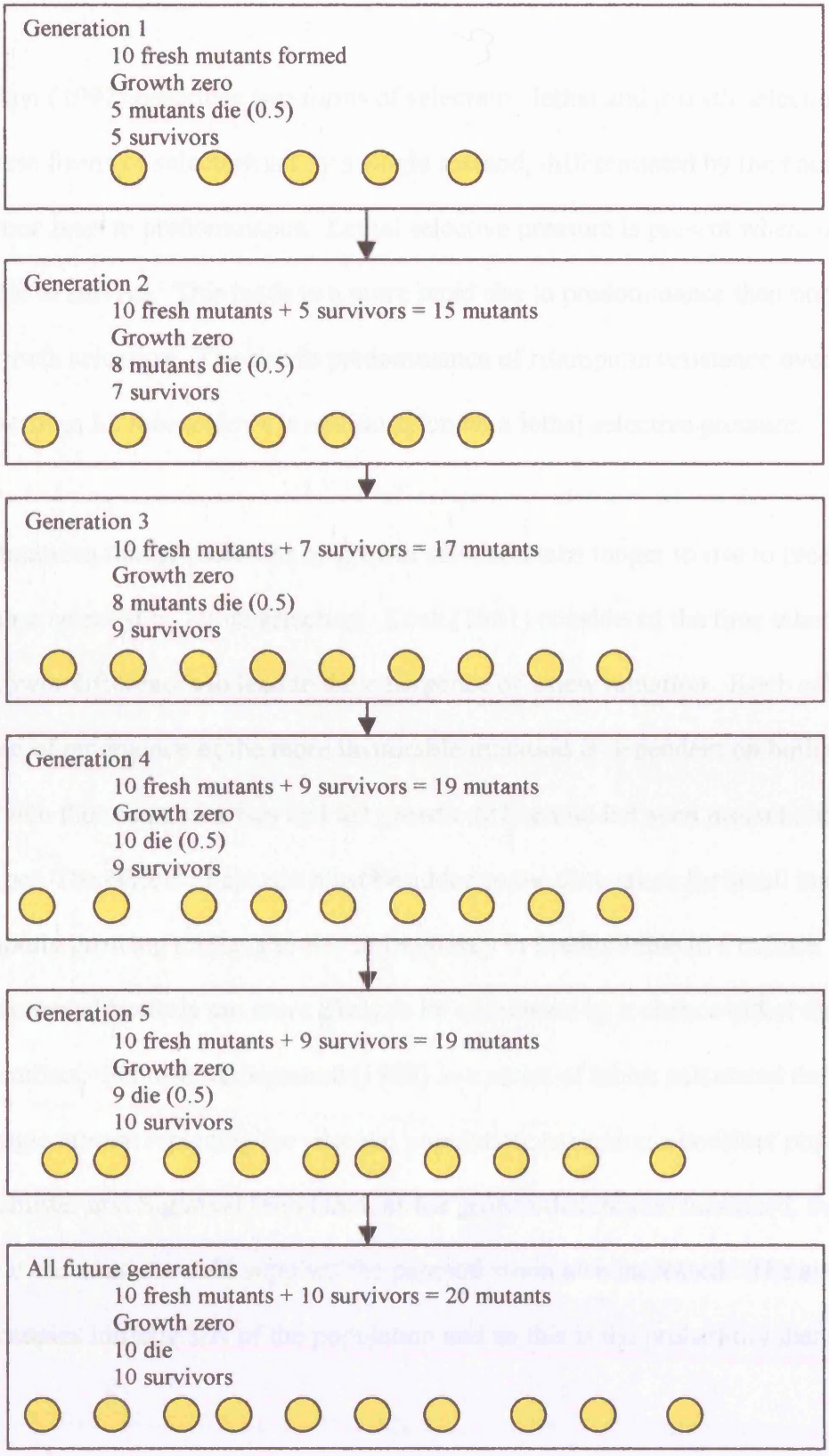
An increase in the growth rate of mutant cells, from either an increased mutation rate of the wild type, or growth of existing mutants, will enlarge the number of freshly created mutants at each generation. To achieve equilibrium this must be matched by a larger number of deaths at each generation. Given a constant death rate of 0.5 per generation in a continuous culture this will produce a larger number of mutant cells forming equilibrium with their parent cell type.

This simple model predicts that, given a large population, a continuous pool of mutant cell types will be available. Drake (1991b) has identified a constant mutation rate of 0.0033 per generation for DNA based organisms. This mutation rate will ensure continuous creation of fresh mutants, although not necessarily the same mutant.

Those mutant types that are both fortunate and relatively rapidly growing compared to the parent cell type may remain as a constant source of phenotypic variation under altering environmental conditions, assuming a large population.

This simple model works only where the mutant cell type has a lower fitness than the parental cell type. The mutant cell type comes to predominate if the relative fitness of the mutant is greater than the parent cell. The mutant frequency will match the parent cell type where the relative fitness of both parent and mutant is the same.

Figure 1.8 Production of a pool of mutants in a continuous culture. Each filled circle represents a single mutant cell.



1.13 The rate of emergence of an advantageous mutation is dependent on the advantage produced by the mutation

Mayr (1997) describes two forms of selection: lethal and growth selection. Both of these forms of selection act by a single method, differentiated by the pace in which one clone rises to predominance. Lethal selective pressure is present where only one clone is able to survive. This leads to a more rapid rise to predominance than non-lethal or growth selection. The rise to predominance of rifampicin resistance over susceptible bacilli in *M. tuberculosis* is obviously under a lethal selective pressure.

Mutations that are selected by growth selection take longer to rise to predominance than those selected by lethal selection. Koch (1981) considered the time taken for small growth differences to lead to the emergence of a new mutation. Koch calculated that the rate of emergence of the more favourable mutation is dependent on both the rate at which the mutation arises and the growth differential between mutant and parental cell type. The effects of chance must be added to the time taken for small numbers of more rapidly growing mutants to rise in frequency to predominate in a culture. A small number of bacteria are more likely to be eliminated by a chance effect than are large numbers. Schuster & Sigmund (1989) in a series of tables calculated the probability of a single mutant replacing the parental population, assuming a constant population size. Schuster and Sigmund found that, as the growth differential increased, the probability that the mutant would supplant the parental strain also increased. The single mutant occupies initially 1/N of the population and so this is the probability that it will be

selected by chance from the population. If the advantageous allele is rejected by pure chance in the first generation it can not then rise to predominance. Schuster and Sigmund describe a 'boundary layer' beyond which an advantageous mutation is not likely to be rejected by chance. The more rapidly the mutant grows, the faster it achieves this 'boundary layer' and so is unlikely to be rejected by chance.

1.14 Does a high mutation rate bring evolutionary advantages?

Taddei, Radman, Maynard-Smith et al. (1997) using mathematical modelling techniques found that cells with increased mutation rates could come to predominate in a population. Cells with high mutation rates were described as mutators. The larger range of variation generated by the mutator strains enable them to rapidly locate favourable mutations. The mutator also created unfavourable mutations, which reduced the genome's fitness and so acted to select against the mutator phenotype. The advantage of an increased mutation rate is limited as both favourable and unfavourable mutations are created, unfavourable mutations being generated far more frequently than favourable mutations. Taddei et al. (1997) described how the mutator phenotype 'hitchhiked' on the selective advantage of advantageous mutations. The mutator phenotype evolved due to selection for the advantageous mutation provided by the wide range of mutation produced by the mutator phenotype, but not directly due to the mutator phenotype. The mutator advantage is at its greatest when there are a large number of possible advantageous mutations and the population spends a long time waiting for these advantageous mutations to arise. Mutators are at a disadvantage when there is either no

or a limited range of advantageous mutations possible and when this produces too high a mutation rate.

Negri, Morosini, Baquero et al. (2002) found that mutator *Streptococcus pneumoniae* produced an advantage in acquiring antibiotic resistance. Two strains of *S. pneumoniae* R310 and R800 were used. Strain R310 had a mutation rate raised approximately 10 times due to mutations in the methyl mismatch repair mechanism. Strain R800 was the wild type with no raised mutation rate. Both strains had the same MIC to cefotaxime 0.008 μg/ml. The mutation rate to cefotaxime resistance was estimated at 3.7×10^{-7} for R310 and 1.13×10^{-8} for R800. Populations of about 2.2×10^{7} CFU/ml were exposed to low concentrations of cefotaxime varying from 0.002 to 0.06 μg/ml for periods of up to 8 hours. The proportion of R310 in the mixed cultures rose as the concentration of cefotaxime increased. After 8 hours of exposure there was no increase in the proportion of the strain R310 for concentrations of cefotaxime below the MIC (0.008 μg/ml), but above this concentration R310 occupied approximately 95% of the surviving population. The surviving populations were identified as having acquired a mutation in the pbp2x gene (Thr550 →Ala), this is associated with resistance to cefotaxime.

Experimental populations not seeded with mutator phenotypes in an environment requiring adaptation have enabled mutation cell types to emerge. Sniegowski, Gerrish & Lenski (1997) cultured *E. coli* in a glucose-limited environment and identified clones with rapid mutation rates coming to predominate in 3 out of 12 populations.

Raised mutation rates have been identified in clinical isolates. LeClerc, Li, Payne et al. (1996) identified one percent of *E. coli* and *Salmonella enterica* with raised mutation rates. This, it was hypothesised, 'may help explain the rapid emergence of antibiotic resistance'. Raised mutation rates have been associated with ciprofloxacin resistance in *E.coli*. The raised mutation rate will have enabled a larger number of mutants to be produced and so enabled mutator bacteria to accumulate ciprofloxacin resistant mutations more rapidly. Lindgren, Karlsson & Hughes (2003) identified a strong correlation between the mutation rate and increasing ciprofloxacin resistance in 54 clinical isolates from patients with uncomplicated urinary tract infections.

It is worth considering the advantage conferred by this raised mutation rate. The probability that a specific mutation is present is dependent on the population size. The average number of a specific mutation (p) with a mutation rate given by μ present is given by the equation $p=N\mu$, where N is the population size. Increases in population size or mutation rate will increase the average number of mutations. If $N\mu$ is equal to 1 then there will on average be one mutation in the population. However, as this is an average there will sometimes be more than one and sometimes no mutations. What is the probability of a mutation being present?

The average number of mutations can be used to calculate the probability of no mutation present. This in turn can be used to calculate the probability of there being at least one mutation, if the average number of mutations is known. The probability that a mutation is absent is calculated from the Po calculation.

Equation 1.3 $Po = e^{-(\mu N)}$

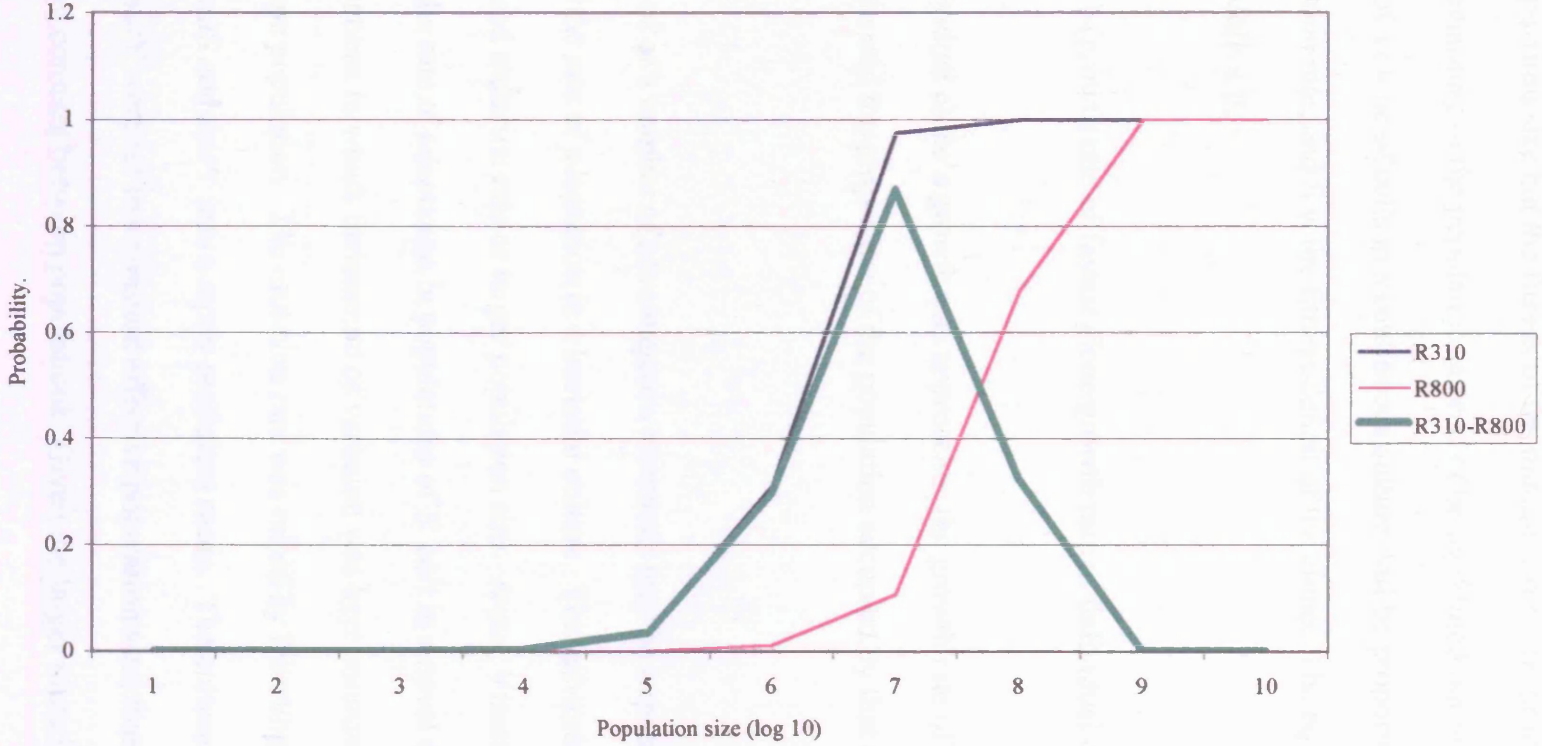
Where Po is the probability of there not being a mutation

μ is the mutation rate and N is the population size.

The probability there is at least one mutation present (Pm) then becomes 1-Po.

A graph of the probability of at least one mutation being present for *S. pneumoniae* mutation rates to cefotaxime based on the rates identified by Negri et al. (2002) for R310 - a mutator strain and R800 - the wild type, at different population sizes, is given in figure 1.9. This graph is a sigmoid curve. The advantage conferred by the high mutation rate being the difference between the two curves and is shown as the curve R310-R800. It is apparent that this advantage has a peak at a population size of 10^7 and this advantage declines at larger or smaller populations. The population size used by Negri et al. (2002) was 2.2×10^7 cfu/ml. A larger population may have enabled the lower mutation rate R800 isolate to produce cefotaxime resistant mutants more frequently. A smaller population would have made the mutation less likely in both strains. This would have reduced, or eliminated, the advantage of the raised mutation rate in R310.

Figure 1.9 Probability of at least one cefotaxime resistant mutant being present in strain R310 and R800 of *Streptococcus pneumoniae* and the difference in probability between these two strains (R310-R800) at different population sizes. Mutation rates taken from Negri et al. (2002)



The probability that a mutation is present in a continuous culture will be dependant not only on population size but the fitness of the mutant and so the probability of that mutation remaining in the population instead of being diluted out of the population. The frequency of a clone of cells in a continuous culture will be proportional to μ/S, where μ is the mutation rate, and S is the fitness deficit of the clone. The equation for calculating the fitness deficit is:

S= 1-(growth rate of fastest clone/growth rate of individual clone).

As the individual clone's growth rate approaches the growth rate of the fastest clone so μ/S increases and the proportion of the population occupied by that clone increases (Bell 1997).

The presence of a number of advantageous mutations may be expected to lead to an increase in the rate of adaptation in a bacterial culture. This advantage is obtainable both by raised mutation rate or larger population size. Arjan, Visser, Zeyl et al. (1999) examined the rate of adaptation in populations of *E. coli* in a novel environment for 1000 generations in which the amount of variation was kept constant in both a mutator and wild type population. The mutation rate was raised by inserting repair deficient copies of *mut*S and *mut*Y into a repair proficient strain. The cultures of both wild type, *mut*S and *mut*Y were set to a constant effective population size, that is the calculation μN was kept constant between populations. Given the larger mutation rates (μ) in the

*mut*S and *mut*Y populations, this meant using a larger total population (N) in the wild type culture at passage. All of the cultures adapted at the same rate regardless of the mutation rate given the same effective population size.

The critical figure is not the mutation rate but the amount of variation. Increasing the mutation rate or the population size can increase the amount of variation.

Genetic drift can enable unfavourable alleles as well as neutral alleles to be adopted. Increased mutation rates increase both favourable and unfavourable mutations This in turn makes mutator cell lines more prone to genetic drift under severe population restriction. Zeyl, Mizesko & de Visser (2001) studied the evolution of *Saccharomyces cerevisiae* for 2900 generations in 12 replicate populations under conditions of genetic restriction. Two of the populations suffered extinction and in both the populations mutator cells came to predominate just prior to extinction. The probability of a less favourable mutation being adopted is dependent on the proportion of the population with less favourable mutations. The presence of too high a variation in combination with severe genetic restriction can lead to a decline in fitness to that environment.

The evolution of drug resistance in *M. tuberculosis* is associated with continuous genetic restriction both in transmission between patients and when the single drug resistant cell is selected. This may produce a restricting factor on the evolution of mutator phenotypes. The large bacilli numbers in pulmonary tuberculosis may mean that there is already a large range of genetic variation in the population so that the mutator phenotype

provides no advantage. The Beijing strain, a widely disseminated strain associated with antibiotic resistance, does not have an increased mutation rate (Werngren & Hoffner 2003).

Jacob (1977) describes selection as not only acting to eliminate detrimental mutations and favouring beneficial mutations, but 'in the long run it orders them (mutations) into adaptively coherent patterns'. The driving force of selection is genetic variation in the population prior to selection.

Nilsson, Berg, Aspevall et al. (2003) identified resistance mutations to fosfomycin, an antibiotic used to treat urinary tract infection (UTI) to be associated with very low fitness. This low fitness resulted in a growth rate 10% to 25% lower than the fosfomycin susceptible strains. The high turn over rate of *E. coli* in the bladder and the low growth rate means that fosfomycin resistance was rapidly eliminated from the population in the absence of fosfomycin. This may explain why fosfomycin resistance is identified at a rate of about 1% of infections in countries both using and not using fosfomycin. This occurs despite calculations by Nilsson et al. (2003) from the rate of mutation rate (10^{-7}) and the bacterial load during UTI ($>10^5$ bacteria per ml) that fosfomycin resistance should be common (10^{-2}). The low fitness of fosfomycin resistance means that variation in resistance is absent prior to exposure to the selective pressure of fosfomycin.

Shimao (1987) did not use the mutation rate but the probability of a mutation being present to identify the probability of drug resistance emerging. This requires knowledge of the effective population (Ne) or the amount of variation that is present in the population. The mutation rate is the generator of that variation but not its sole determinant. The mutation rate can also be dependant on the environment. The presence of mutagenic compounds will raise the mutation rate. Such mutagenic chemicals surround *M. tuberculosis* particularly in those populations inhabiting the inflammatory or necrotic loci, Boshoff, Durbach & Mizrahi (2001) outline many of these factors. This indicates that the mutation rate assessed *in vitro* may not be as high as that identified *in vivo* especially where the bacterium is being damaged by a successful immune response.

1.15 Essential mutations carry a physiological cost, this can be compensated for by other mutations

The basis of antibiotic policy is that variation of selective pressure will retard the emergence of antibiotic resistance (Levin et al. 1997). There is some debate as to whether antibiotic resistance carries with it a concomitant physiological cost (Gillespie & McHugh 1997).

There is evidence both for and against this physiological cost in *M. tuberculosis*. The reversion to susceptibility of streptomycin resistant *M. tuberculosis* suggests a physiological cost (British Medical Council 1950). However, isoniazid resistant *M.*

tuberculosis does not revert despite an apparent virulence advantage in susceptible *M. tuberculosis* (Medical Research Council 1953b). Isoniazid susceptible strains can accumulate but not come to predominate (Fox, Ellard & Mitchison 1999).

The presence of compensatory mutations is evidence for a physiological cost. Compensatory mutations ameliorate the physiological cost of a mutation without reversing the phenotypic alteration. An example of this is streptomycin resistance arising from mutation in the *rps*L gene. This gene codes for the ribosomal protein S12 and so can affect the rate of protein synthesis. Schrag & Perrot (1996) adapted *E. coli* resistant to streptomycin by continuous culture of a clone with a mutation in the *rps*L gene. The non-adapted strains of *E.coli*, fresh streptomycin resistant mutants, were less able to grow in competition with susceptible strains. This growth inhibition was reflected in less rapid protein synthesis. The adapted strains of *E. coli* had a reduced phenotypic cost associated with streptomycin resistance and protein was synthesised at a faster rate (see table 1.5).

Table 1.5 Adaptation to streptomycin resistance in *E. coli* based on the work of Schrag & Perrot (1996). The reduction in fitness deficit with adaptation is accompanied by an increased peptide chain elongation rate.

Strain	Evolutionary status	DNA sequence at amino acid position 42 of the *rps*L gene	Cost of resistance (% per generation ± S.E)	Peptide chain elongation rate (amino acid per second, ± S.E)
STR1	Baseline	ACA (Thr)	13.6 ± 0.57	10.60 ± 0.70
STR1-1	Evolved	ACA (Thr)	6.97 ± 0.6	17.54 ± 1.1
STR1-2	Evolved	ACA (Thr)	4.63 ± 0.55	17.93 ± 1.6
STR2	Baseline	AAC (Asn)	18.8 ± 0.79	8.74 ± 0.56
STR2-1	Evolved	AAC (Asn)	9.55 ± 1.3	18.45 ± 1.59
STR2-2	Evolved	AAC (Asn)	6.68 ± 0.51	18.47 ± 1.77

The adaptations to antibiotic resistance need not be of advantage in the susceptible genetic background. Isoniazid resistance is associated in *M. tuberculosis* with loss of the catalase enzyme (Zhang, Dhandayuthapani & Deretic 1996).

Mutations in the *ahp*C gene are reported as associated with loss of the *kat*G gene (Kelley, Rouse & Morris 1997; Rinder, Thomschke, Rusch-Gerdes et al. 1998). Sherman, Mdluli, Hickey et al. (1996) identified a higher susceptibility of *M. bovis* BCG lacking the *kat*G gene to hydrogen peroxide: the *kat*G gene product is catalase, an enzyme that converts hydrogen peroxide to water and oxygen, and so protects the cell from hydrogen peroxide. This indicates that resistance to isoniazid may make *M. tuberculosis* more susceptible to the oxidative burst of the macrophage.

The *ahp*C gene codes for alkyl hydroperoxide reductase, over production of the gene product *ahp*C can help protect against hydrogen peroxide. This over production of alkyl

hydroperoxide reductase entails a physiological cost. This physiological cost will inhibit the ability of resistant strains to compete in the absence of hydrogen peroxide. This cost need not be borne by an isoniazid susceptible strain retaining catalase activity in the absence of isoniazid. Compensatory mutations in the *ahp*C gene enhance the fitness of the isoniazid resistant cell but not the fitness of an isoniazid susceptible cell.

Lenski (1988) studied the adaptation of *E. coli* to the selective pressure created by a plasmid. The initial insertion of the plasmid led to a decline in the ability of *E. coli* to compete with *E. coli* lacking the plasmid. This physiological cost declined on passage of *E. coli* in the presence of the plasmid. Lenski then removed the plasmid from the *E. coli* clone adapted to the presence of the plasmid. This removal of the plasmid lead to a decline in the cell's relative fitness. Lenski concluded that the cell adapts to its total environment and alteration in that environment, including alteration of accompanying genes, affects the cell's ability to compete.

The growth of haploid cells with no means of genetic exchange means that all cells effectively create their own separate lineage of cells. These different lineages must then compete on their ability to grow. Separate advantageous mutations arising on separate lineages must compete with only the most fortuitous or best-adapted rising to predominance.

The continuous growth of haploid cells in the same genetic environment enables a strong linkage between genes to develop. Bell (1997) states that linkage between genes such as

pleotophy, where a single gene can affect a number of characters and epistasis where two or more genes affect the combined production of fitness, is increased in haploid cells. Each gene will have evolved in the same environment and optimal characters for that genetic environment will be produced.

The development of antibiotic resistance may or may not carry a physiological cost. Antibiotic resistance that compromises a virulence factor will obviously reduce the virulence of a bacillus, however MDR-TB is capable of causing disease (Department of Health 1998b; Pablos-Mendez et al. 1998). The development of a physiological cost, although not a recognised virulence determinant, will affect that clone's ability to grow.

Selection can discriminate between cells with very small differences in fitness. The cell has evolved a characteristic G.C ratio and preferential usage of specific codons. The G.C ratio varies between cells. Insertion sequences and pathogenicity islands are recognisable from the alteration in G.C ratio compared to other genome contents (Groisman & Ochman 1996). The time since insertion can be estimated from the degree of variation in G.C ratio from the other genome contents. This indicates a selective pressure, selecting those mutations that lead the pathogenic island to contain a G.C ratio closer to the normal cell ratio. A very small loss of fitness associated with antibiotic resistance will over time affect the likelihood of that resistance remaining in a bacterial population.

The codons in rapidly produced genes characteristically are those in which the cell has most tRNA molecules. The time delay introduced by codons for unusual or low frequency tRNA molecules introduce a slight physiological cost in the delayed translation of a gene (Sharp, Stenico, Peden et al. 1993; Wright 1990).

The adaptations of G.C ratio and codon usage in the genome indicate that adaptation is capable of differentiating very small differences in the growth abilities of haploid cells.

1.16 Fitness estimates of isolates with drug resistance in *M. tuberculosis*

Reversion to susceptibility of drug resistant isolates in the absence of the drug is evidence that resistance has an associated disadvantage. The earlier trials with streptomycin and PAS identified that *M. tuberculosis* that became streptomycin resistant during treatment had high rates of reversion to streptomycin susceptibility if treatment was stopped. This reversion was identified as due to the higher growth rates of susceptible strains. The higher growth rate allowed any surviving streptomycin susceptible clones to out-compete a resistant strain in the absence of streptomycin (Mitchison 1950). Streptomycin resistance is still common. Pablos-Mendez et al. (1998) reported that the median frequency of mono *M. tuberculosis* streptomycin resistant world-wide was 2.5%.

There is some evidence that isoniazid resistance can reduce the virulence of *M. tuberculosis* in guinea pigs. Middlebrook and Cohn (1953) found that animals infected

with a strain of H37Rv isoniazid resistant survived for over 60 days. Guinea pigs infected with H37Rv isoniazid sensitive died between 19 and 25 days after infection. The isoniazid resistant strain lacked detectable catalase activity. Isoniazid is activated by the activity of catalase, however as discussed earlier, this is also a defence against the macrophage oxidative burst. Li, Kelley, Collins et al. (1998) infected BALB/c mice and guinea pigs intravenously with H37Rv both lacking catalase activity and with catalase activity. The catalase lacking H37Rv was unable to persist in the spleens of the mice or guinea pigs. Transformation of the H37Rv lacking catalase activity with the catalase gene from either *M. tuberculosis* or *M.intracellulare* returned catalase activity and the ability to persist in the spleen of mice and guinea pigs. Transformation with an *M. tuberculosis* catalase gene with the mutation T275P returned only partial catalase activity. *M. tuberculosis* H37Rv strains with the mutation T275P were unable to persist in the guinea pig spleen, although they were able to persist in mouse tissues.

Isoniazid resistant strains of tuberculosis failed to revert to susceptible on removal of selective pressure (Medical Research Council 1953a; Medical Research Council 1954). This failure to revert to susceptible occurred despite *M. tuberculosis* with high resistance to isoniazid having a reduced virulence in guinea pigs. The failure of isoniazid resistance to revert to susceptible in the absence of antibiotic could be explained if susceptible strains were entirely absent. The use of isoniazid in combination with thiacetozone did produce favourable responses in at least 10 out of 30 patients with primary isoniazid resistant tuberculosis infection. The response of patients with primary isoniazid resistant tuberculosis to isoniazid in the first 3 months was not related to the

MIC of the infecting strain, but the response was related to the amount of growth on the slope containing 0.2 ug Isoniazid per ml. Fox et al (1999) state this favourable response to isoniazid implies at least a portion of the infecting tuberculosis had reverted to susceptibility to isoniazid.

Transmission of *M. tuberculosis* with primary isoniazid resistance has been well reported. This leads to the suggestion that the difference in virulence in the guinea pig was an experimental artefact. However there is evidence of a decreasing isoniazid MIC in transmitted primary resistant tuberculosis (Fox, Ellard & Mitchison 1999).

Cohen, Sommers & Murray (2003), in a review of the effect of drug resistance on the fitness of *M. tuberculosis*, found that population based studies of fitness in MDR-TB produced a wide variation in fitness. From this variation he concluded that the fitness of *M. tuberculosis* is very heterogeneous and 'this variation may preclude our ability to predict future trends of this pathogen'. This implies that genetic drift has a major role in *M. tuberculosis* evolution and that the differential reproductive ability or infectivity of strains of *M. tuberculosis* and the selective pressure this exerts will have less effect than the role of chance. I intend to address the roles of genetic drift and selection in the evolution of drug resistance in *M. tuberculosis* to test whether there is a role for selection.

Chapter 2

Methods

2.1 Maintenance, storage and bacteria strains used

Isolates were maintained by growth at 37°C on Löwenstein Jensen media (Southern Group Media), 7H9 broth (0.1% tween 80, Difco, Hemel Hempstead, United Kingdom) BACTEC 12B media (Becton Dickinson, Oxford, United Kingdom) or growth on Middlebrook 7H10 agar. Isolates were stored by freezing at –20°C in fresh 7H9 broth. All culture work was carried out at the Royal Free Hospital under category 3 containment safety rules (Health and Safety Executive 2002).

2.1.1 Isolates used:

2.1.1.a *M. tuberculosis* H37Rv ATCC 9360 (National Collection of Type cultures, Central Public Health Laboratory, London, United Kingdom).

2.1.1.b Strains ET and AT are isolates from a pair of related patients. The isolates have an identical PGRS and IS6110 typing patterns (Davies, Billington, Bannister et al. 2000a). Full details given are given in chapter 5.

2.1.1.c Three isolates from a patient who was the source of an outbreak of MDR-TB in a London Hospital were obtained (Breathnach, de Ruiter,

Holdsworth et al. 1998). One isolate from each of two patients who were a part of the outbreak was also obtained. The isolates from the source patient were labelled S-1, S-2 and S-Brompton. The isolate S-Brompton was obtained from the Brompton Hospital. The isolates from the two patients in the outbreak were labelled X and Y.

2.2 DNA extraction

To extract DNA from *M. tuberculosis* 1-ml of Middlebrook 7H9 broth culture was centrifuged (12,000-x g for 5 minutes) in a 1.5-ml microcentrifuge tube, and the supernatant discarded. The deposit was heated to 80°C for 20 minutes in a water bath to kill all *M. tuberculosis*. An aliquot of 100 µl of chloroform was added to the deposit and vortexed for 30 seconds. The sample was then centrifuged 12,000 g for 1 minute and the aqueous layer was retained for use in the subsequent PCRs (Wilson, McNerney, Nye et al. 1993).

2.3 *rpo*B Polymerase Chain Reaction methods (PCR)

Two PCR reactions were designed around the *rpo*B gene: PCR-120 which produced a PCR product 120 bp long and was used for SSCP-PCR analysis and PCR-411 which produced a PCR product 411 bp long and was used for sequencing. Telenti et al. (1993b) sequenced the *rpo*B gene and PCR-120 and PCR-411 were designed based on this sequence.

Telenti et al. (1993a) identified the *rpo*B hot spot leading to rifampicin resistance. Ramaswamy & Musser (1998) described the hot spot as 81 bp long between codons 507-533. Both PCR-120 and PCR-411 were designed to amplify this region. The sequence of this region is given in figure 2.1. The two positions where mutation is most frequently clinically isolated leading to rifampicin resistance are marked with an asterisk.

A separate PCR product was required for SSCP and sequencing. SSCP requires a short stretch of DNA, which can not then be reliably sequenced. SSCP detects small changes in the conformation of single stranded DNA. As the DNA reanneals the folding is dependent on the formation of intramolecular bonds. Hayashi (1991) recommends a PCR product of less than 400 bp and Telenti et al. (1993b) produced a reliable result from PCR-SSCP of a PCR product 157 bp long from this region of the *rpo*B gene.

2.3.1 Polymerase Chain Reaction and Single Strand Conformation Pattern (PCR-SSCP)

The region of the *rpo*B gene identified previously by Telenti et al. (1993a) as a hot spot for rifampicin resistance was amplified by PCR. The primers employed were 5'-AGT TCT TCG GCA CCA GC-3' and 5'-CGC TCA CGT GAC AGA CC-3'. The optimum reaction mixture was 1.5 nM. MgCl, 150-mM deoxynucleoside triphosphates, 5 U of Taq polymerase enzyme, and 500 μM primers for each PCR in a total volume of 90 μl. An aliquot of 10 μl of the *M. tuberculosis* DNA extract was added. The optimal PCR cycling conditions were one cycle 95°C for 1 minute and 30 cycles of 94°C for 1

minute, 65°C for 2 minutes and 72°C for 3 minutes. This was followed by strand elongation for 7 minutes at 72°C. This produced a 120 base pair amplimer that was designated PCR120. The size of the PCR product was confirmed by running a 10ul aliquote of the PCR product on a 1.5-% agarose gel containing ethidium bromide to make the DNA visible under UV light. The size of the PCR product was estimated from comparison with a DNA ladder composed of phage λDNA.

The amplimers were analysed by single strand conformation polymorphism (SSCP) analysis. The PCR120 product (6μl) was denatured at 95°C for 10 minutes with 3μl of SSCP loading buffer and 3μl of stop dye (Promega, Southampton, United Kingdom). Samples were quenched on ice and then loaded directly onto a 0.5% mutation detection enhancement acrylamide analogue gel (Flowgen, Lichfield, United Kingdom). The gel was run for 6 hours at 6 watts with 0.6 x Tris-borate-EDTA (Sigma, Poole, United Kingdom) at room temperature. DNA bands were visualised by the silver-staining technique, following the manufacturers instructions (Promega).

2.3.2 Polymerase Chain Reaction methods (PCR) for sequence analysis of the *rpo*B gene

An ampilimer 411 bp long was prepared for direct sequence analysis, and this amplimer was designated PCR411. The primers published by Telenti et al. (1993a) 5'- TAC GGT CGG CGA GTG ATC AA-3' and 5'-TAC GGC GTT TCG ATG AAC C-3' were used; the optimised reaction mixture was 50 mM KCl buffer, 1.5 mM MgCL, and 150 mM deoxynucleoside triphosphates, and 5 U of *Taq*. The cycling conditions described for

the 120bp amplimer were also used in this reaction. These primers amplified a 411-bp fragment. The size of the PCR product was confirmed by running a 10ul aliquote of the PCR product on a 1.5-% agarose gel containing ethidium bromide to make the DNA visible under UV light. The size of the PCR product was estimated from comparison with a DNA ladder composed of phage λDNA. The PCR product was sent for sequencing as in section 2.6.

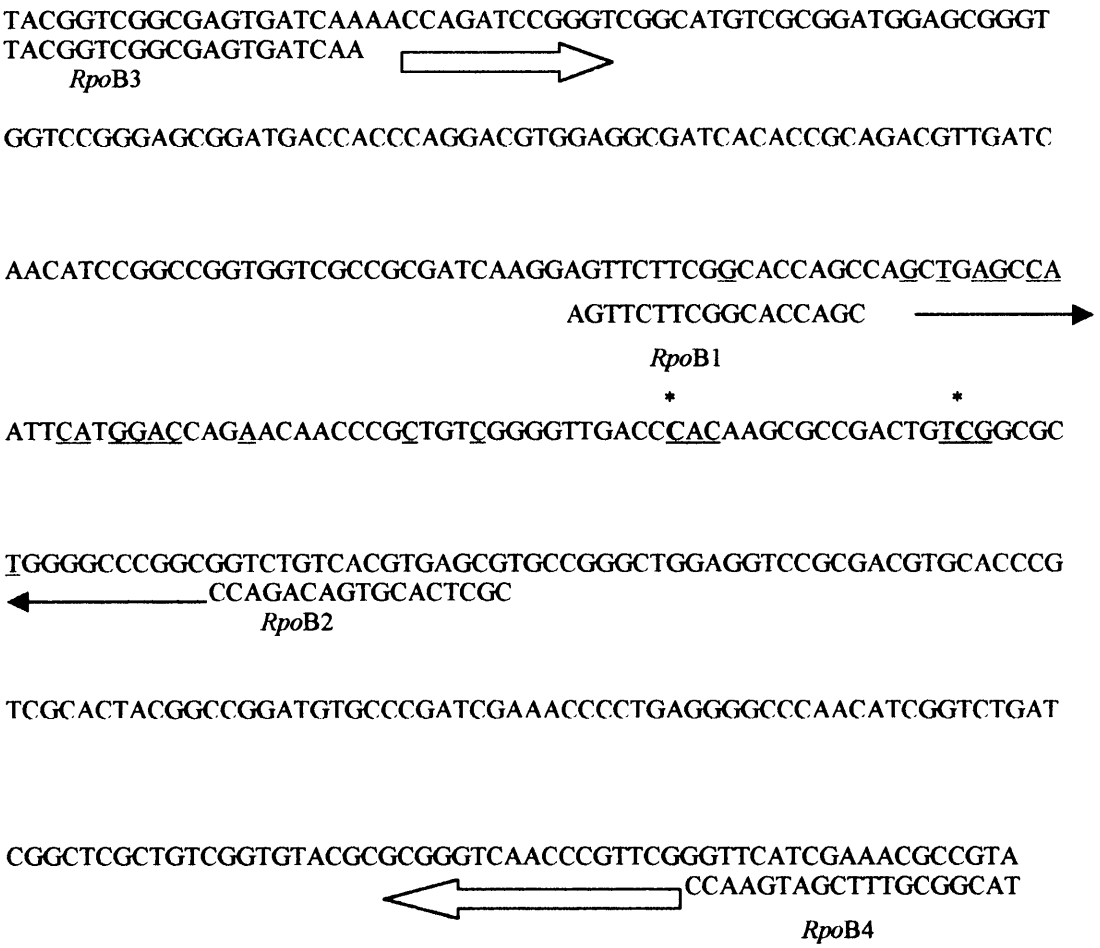


Figure 2.1 The *rpo*B gene amplified by PCR-411 (between primers *Rpo*B3 and *Rpo*B4) and PCR-120 (between PCR primers *Rpo*B1 and *Rpo*B2). The bases underlined are those described by Ramaswamy and Musser (1998) where point mutation leads to rifampicin resistance. An asterisk is above the two bases where mutation leading to rifampicin resistance is most frequently clinically isolated.

2.4 *pncA* Polymerase Chain Reaction methods (PCR)

The *pncA* gene encoding pyrazinamidase (PZAase) described by Scorpio & Zhang (1996) was amplified by PCR. The primers employed were 5'-TGC GGG CGT TGA TCA TC 3' AND 5'CAG GAG CTG CAA ACC AAC TC-3'. The optimal reaction mixture contained 1.5mM MgCl in KCl buffer, 150 mM. deoxynucleoside triphosphates, and 5 U of *Taq* polymerase. The optimal PCR cycling conditions were one cycle 95°C for 1 minute and 35 cycles of 94°C for 1 minute, 55°C for 2 minutes and 72°Cfor 3 minutes. This was followed by strand elongation for 7 minutes at 72°C.

The product of the PCR was an amplimer of 559bp. The size of the PCR product was confirmed by running a 10 µl aliquote of the PCR product on a 1.5 % agarose gel containing ethidium bromide to make the DNA visible under UV light. The size of the PCR product was estimated from comparison with a DNA ladder composed of phage λDNA. The PCR product was sent for sequencing as in section 2.6.

ATGCGGGCGTTGATCATCGTGACCTGCAGAACGACTTCTGCGAGGGTGGCTC

TGCGGGCGTTGATCATC ⟹

GCTGGCGGTAACCGGCGGCGCCGCGCTGGCCCGCGCCATCAGCGACTACCTG

GCCGAAGCGGCGGACTACCATCACGTCGTGGCAACCAAGGACTTCCACATCG

ACCCGGGTGACCACTTCTCCGGCACACCGGACTATTCCTCGTCGTGGCCACC

GCATTGCGTCAGCGGTACTCCCGGCGCGGACTTCCATCCCAGTCTGGACACG

TCGGCAATCGAGGCGGTGTTCTACAAGGGTGCCTACACCGGAGCGTACAGCG

GCTTCGAAGGAGTCGACGAGAACGGCACGCCACTGCTGAATTGGCTGCGGCA

ACGCGGCGTCGATGAGGTCGATGTGGTCGGTATTGCCACCGATCATTGTGTG

CGCCAGACGGCCGAGGACGCGGTACGCAATGGCTTGGCCACCAGGGTGCTG

GTGGACCTGACAGCGGGTGTGTCGGCCGATACCACCGTCGCCGCGCTGGAGA

TGCGCACCGCCAGCGTCGAGTTGGTTTGCAGCTCCTGC

⟸ *CTCAACCAAACGTCGAGGAG*

Figure 2.2 The pyrazinamidase/nicotinamidase gene described by Scorpio & Zhang (1996). The primers used are given in italics beneath the DNA sequence.

2.5 DNA gyrase polymerase chain reaction methods

2.5.1 *gyr*A PCR

Primers were 5'-ATC GCC GGG TGC TCT ATG C-3' and 5'-GTG GGT CAT TGC CTG GCG A-3'. The optimal reaction mixture contained 0.1 μM of each primer, 5 mM dNTPs, 50 mM KCl, 15 mM $MgCl_2$ and 1 unit *Taq* polymerase enzyme (Bioline, London UK).

The cycling conditions were 1 cycle 94°C for 1 min to ensure single stranded DNA. Amplification was by 30 cycles of 94°C for 1 min, 50°C for 2 min and 72°C for 3 min. The amplification cycles were followed by a strand elongation step of 7 min at 72°C.

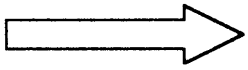
The product of the PCR was an amplimer of 216 bp. The product was sent for sequencing as in section 2.5. This PCR was designed and optimised by Shreya Basu.

2.5.2 *gyr*B PCR

Primers were 5' GAA GGC CCA AGA CGA 3' and 5' GAC TTC GAG CCG GGG TGG ATA 3'. The optimal mixture contained 0.1 μM of each primer, 5 mM dNTPs, 10 mM NH_4PO_3, 1.5 mM $MgCL_2$ and 1 unit *Taq* polymerase enzyme (Bioline, London UK). This PCR was designed and optimised by Shreya Basu.

The cycling conditions were 1 cycle 94°C for 1 min to ensure single stranded DNA. Amplification was by 30 cycles of 94°C for 1 min, 60°C for 2 min and 72°C for 3 min. The amplification cycles were followed by a strand elongation step of 7 min at 72°C.

The product of the PCR was an amplimer of 394 bp. The product was sent for sequencing as in section 2.6. This PCR was designed and optimised by Shreya Basu.

atcgccgggtgctctatgcaatgttcgattccggcttccgcccggaccgcagccacgccaagtcggcccgg
atcgccgggtgctctatgc 

tcggttgccgagaccatgggcaactaccacccgcacggcgacgcgtcgatctacgacagcctggtgcgca

tggcccagccctggtcgctgcgctacccgctggtggacggccagggcaacttcggctcgccaggcaatga
gcggaccgttact

cccac
gggtg

Figure 2.3a The section of the *gyr*A gene amplified by the primers used. The primers used are illustrated beneath the sequence. The DNA sequence is from Takiff, Salazar, Guerrero et al. (1994).

gaaggcccaagacgaatacggcgctgcgtctatcaccattctcgaagggctggaggccgtccgcaaacgt
gaaggcccaagacga

cccggcatgtacattggctcgaccggtgagcgcggtttacaccatctcatttgggaggtggtcgacaacgcg

gtcgacgaggcgatggccggttatgcaaccacagtgaacgtagtgctgcttgaggatggcggtgtcgaggt

cgccgacgacggccgcggcattccggtcgccacccacgcctccggcataccgaccgtcgacgtggtgatg

acacaactacatgccggcggcaagttcgactcggacgcgtatgcgatatctggtggtctgcacggcgtcgg

cgtgtcggtggttaacgcgctatccacccggctcgaagtc
ataggtgggccgagcttcag

Figure 2.3b The section of the *gyr*B gene amplified by the primers used. The primers used are illustrated beneath the sequence. The DNA sequence is from Takiff et al. (1994).

2.6 Sequencing polymerase chain reaction products

PCR products were purified using the Wizard PCR cleaning kit following the manufacturer's instructions (Promega, Southampton UK). Sequencing was performed commercially by cycle sequencing with dye technology (Cambridge Biosciences, Cambridge, UK).

2.7 Miles and Misra plate count

This is a viable count technique devised by Miles & Misra (1938) in which the number of colony forming units (CFU) produced from a series of small drops of broth diluent are counted.

M. tuberculosis was cultured in Middlebrook 7H9 broth containing tween 80 (0.1%) to reduce clumping. The broth culture was passed 8 to 10 times through a 1-ml fine-needle syringe (28-gauge Sherwood, Crawley, United Kingdom) prior to dilution or subculture.

The dispersed broth was serially diluted 1:10 in tween-albumin broth (0.1% tween 80, Difco, Hemel Hempstead, United Kingdom) and 0.2% bovine albumin (Sigma, Poole, United Kingdom) to form a dilution series from 10^{-1} to 10^{-7}. The tween albumin broth was briefly vortexed three times prior to sampling. At least three samples of 50ul were placed onto the surface of a Middlebrook 7H10 agar plate.

The agar plates were dried at 37°C for 15 minutes prior to inoculation. The plates were incubated agar surface up for 48 hours at 37°C to allow the drops to soak into the agar. The plates were then incubated at 37°C and over 2 to 4 weeks the numbers of colonies were counted for each drop.

The frequency of colonies growing in a viable count technique will follow a Poisson distribution (Meynell & Meynell 1970). This means the number of organisms counted, not the number of repeated counts, determines the accuracy of a count. The standard error being $\sqrt{N}$ of a count of N organisms. The counts in this work were in the region of between 100 CFU giving a standard error of 10 and 95% confidence limits of approximately 20% of the count. Counts were only acceptable if at least 50 CFU were present.

2.8 Fitness assays

The relative fitness of drug resistant *M. tuberculosis* compared to a susceptible strain was determined by the ratio of generations produced by the two strains in a mixed culture.

The number of bacteria present in a broth was determined using a Miles and Misra plate count (section 2.7). Plate counts on drug free Middlebrook 7H10 agar gave the total number of bacteria (T) present in a broth. Plate counts on Middlebrook 7H10 agar with

5 mg/L rifampicin gave the number of drug resistant bacteria (R). The number of susceptible bacteria (S) could then be calculated from formula 2.1

Formula 2.1 S=T-R.

The number of generations (G) of bacteria that had been produced in a broth was calculated from formula 2.2.

Formula 2.2 $$G = \frac{\text{Log Nt} - \text{Log No}}{\text{Log 2}}$$

Where Nt is the number of bacteria present at the end of growth.

No is the number of bacteria inoculated at the start of growth.

The fitness of the multi-drug resistant strain was determined using formula 2.3

Formula 2.3 Fitness = Generations of MDR-TB / Generations of fully sensitive TB.

The accuracy of a fitness estimate will be in part determined by the Miles and Misra plate count (see section 2.7). The accuracy will also be dependent on the calculation of the number of susceptible organisms in the mixed broth using formula 2.1. There is an upper and lower 95% confidence limit to counts on both drug free and drug containing agar. An estimate for the upper and lower counts for the number of susceptible colonies present can then be identified from the worst cases of these two estimates see formula 2.4 and 2.5

Formula 2.4 Upper limit of sensitive count $S_u = T_L - R_u$

Formula 2.5 Lower limit of sensitive count $S_L = T_u - R_L$

Where S_u and S_L are the upper and lower limits of sensitive CFU estimate.

T_u and T_L are the upper and lower limits of total CFU estimate.

and R_u and R_L are the upper and lower limits of resistant CFU estimate.

The error of the sensitive estimate is magnified by the size of the estimated number of resistant organisms present. The larger the number of resistant organisms the larger the error in the estimated number of sensitive colonies. If the numbers of resistant organisms are too great then the number of colony forming units on drug free and drug containing plates become the same.

To determine the relative fitness of resistant *M. tuberculosis* a larger number of sensitive organisms were used in the inocula of the mixed broths. The very much smaller number of drug resistant organisms meant that the error in calculated sensitive organisms was determined by variation in the drug free plate count alone.

2.9 Ciprofloxacin susceptibility

The ciprofloxacin susceptibility test was that recommended by the manufacturer of the Bactec 460.

Two pH 6.4 vials were prepared one containing 1ug/ml ciprofloxacin. A 0.5 McFarland broth culture of *M. tuberculosis* was prepared in Middlebrook 7H9 broth. This broth culture was diluted 1:40 in Middlebrook 7H9 broth and 0.1-ml of the dilute culture inoculated into the vial containing 1ug/ml ciprofloxacin. The broth culture was then diluted 1:100 in tween albumin broth and 0.1 ml of this dilution inoculated into the drug free vial.

The two vials were read daily and the growth index recorded. Susceptibility was recorded if on the day that the drug free control read greater than 30, the change on that day in the growth index for the drug-containing vial was less than the change in the control vial. Resistance was recorded if the change in the drug-containing vial was greater than the control vial, or the drug-containing vial had reached a growth index of 999.

A fully susceptible control H37Rv was set up with each batch of sensitivity tests to confirm activity of the antibiotic.

2.10 Pyrazinamide susceptibility

The pyrazinamide susceptibility test was that recommended by the manufacturer of the Bactec 460. Two vials were prepared one containing 100-ug/ml pyrazinamide the second being drug free, both vials at a ph of 6.0. An equal size bacteria inocula was made into both vials. The vials were incubated at 37°C and read daily on the Bactec 460 and the growth values recorded. Resistance to pyrazinamide was recorded when the drug free vial read >200 if the drug vial was >11% of the drug free vial. Susceptibility was recorded if when the drug free vial was reading 200 the drug containing vial was <9% of the drug free vial. The test was repeated if the drug free vial failed to show a growth index of >200 in 10 days, or reach a growth index of >200 in less than 4 days. The test was also repeated if the drug vial was between 9 and 11% of the drug free vial when a growth index of 200 was reached in the drug free vial.

2.11 Measuring the mutation rate

Crane, Thomas & Jones (1996) described a simple formula, which enables the mutation rate to be determined from an estimation of the median frequency of mutants where the total broth was not sampled. This formula was used in chapter 3 to determine the mutation rate of each individual mutant type leading to rifampicin resistance. The formula was used for this purpose as only a sample of the rifampicin resistant mutants need be typed from each broth culture. The formula of Crane et al. (1996) is given in formula 2.6.

Formula 2.6 $M_e = (T_m - 0.693)/(\ln T_m + 0.367)$.

Where Me is the average number of mutation events which have occurred in per broth and Tm is the median frequency of mutants identified in that series of broths.

The mutation rate (μ) is then calculated from formula 2.7

Formula 2.7 $\mu = M_e$/ median broth total colony forming units.

The aim of determining the mutation rate in chapter 3 is to identify whether rifampicin resistant alleles are formed at different rates. The mutation rates are not directly compared, mutation rates are calculated from the frequency of mutants, calculation of their similarity would require a knowledge of the standard deviation of these estimates.

To identify if more mutants are able to grow at 5 mg/L than 10 mg/L rifampicin, it is necessary only to test that in each broth any difference in colony count on rifampicin containing media, is determined by chance. The number of cells sampled from a single broth will follow a Poisson distribution, variation in sampling alone being tested. This distribution allows tests based upon the normal distribution to be used. The matched pair t test is used in chapter 3 to compare the frequency of mutants isolated at two antibiotic concentrations (5mg/L and 10 mg/L).

The frequency of *rpo*B mutation types required frequencies from a number of different broths to be compared. The numbers of these mutants will be described by the Luria-Delbrück distribution. This is not a normal distribution and statistical tests based on the normal distribution can not be used. The Mann-Whitney U test a non-parametric test is applicable to this data and is recommended for comparison of mutant frequencies (Rosche & Foster 2000).

Chapter 3

The mutation rate of *rpo*B mutations leading to rifampicin resistance *in vitro*

3.1 Introduction

Antimicrobial drug resistance arises during tuberculosis infection either by primary infection with a drug resistant strain or from acquired drug resistance. Acquired resistances to anti-tuberculosis agents in *M. tuberculosis* arise solely by chromosomal mutation and selection under antibiotic pressure (Levin et al. 1997; Livermore 1998; Mitchison 1998; Gillespie 2002). Rifampicin resistance arises from mutation in a small (81 bp) region of the *rpo*B gene (Telenti et al. 1993). No transferable genetic elements have been described that can carry antibiotic resistance genes in *M. tuberculosis* (Bjorkman, Hughes & Andersson 1998; Blanchard 1996; Cole 1994).

In each patient in whom drug resistant *M. tuberculosis* evolves, the infecting strain of bacteria must develop resistant mutants and antibiotic selective pressure then drives these mutants into predominance.

Shimao (1987) proposed a calculation to give the probability of multiple antimicrobial agent resistance arising simultaneously by spontaneous mutation. This required determining the frequency at which resistance to each individual antimicrobial agent arose. The probability that resistance to both antimicrobial agents arose at the same time was determined by multiplying these individual probabilities together. As an example

the frequency of rifampicin resistant mutants arising was determined as 10^{-8} and isoniazid resistance as 10^{-6} in a fully susceptible unselected population. Therefore the probability of both mutations occurring simultaneously in the same bacilli is $10^{-8}x10^{-6}=10^{-14}$. Shimao assumed that in a pulmonary cavity where there are 10^{8} bacilli the probability of resistance to both rifampicin and isoniazid arising simultaneously is 10^{-6}.

Shimao (1987) derived the probability of a mono-drug resistant mutant being present from the frequency of drug resistant bacilli in a broth culture (with units of drug resistant cells per susceptible cell). This frequency, as outlined in the introduction, is dependent on the size of broth used to determine the mutant frequency (section 1.10). Mutation rates are independent of broth size. David (1970) measured the mutation rate to resistance and produced figures of 10^{-8} and 10^{-10} for isoniazid and rifampicin respectively (see table 3.1). The probability of a double mutation, leading to both rifampicin and isoniazid resistance, occurring in a single generation can then be calculated from David's mutation rates as 10^{-18} per bacteria per generation.

Mitchison (1998) stated that the general model for the emergence of drug resistance in *M. tuberculosis* is due to sequential effective monotherapy. Mono-resistance to rifampicin is very rare and is associated with use of rifamycins with prolonged serum half lives and malabsorption, possibly leading to altered uptake and altered pharmokinetics of antituberculosis agents (Munsiff, Joseph, Ebrahimzadeh et al. 1997; Sandman, Schluger, Davidow et al. 1999; Weiner, Burman, Vernon et al. 2003).

The low frequency of rifampicin mono-resistance and far higher frequency of streptomycin and isoniazid mono-resistance is noticeable (see table 3.1). Mitchison (1998) commenting on British Medical Research Council relapse cultures states: 'The complete absence of resistance to rifampicin alone is noteworthy, probably because mutants to rifampicin are much less common than those to isoniazid in a sensitive bacterial population.' The association of rifampicin mono-resistance with specific clinical conditions and the low mutation rate to form rifampicin resistance, indicates that the mutation rate may form a possible limiting factor. This implies there is an absence of available mutations to be selected during therapy. Evolution can only operate on those alleles actually present. Through a complete cell cycle a population of genetically identical bacteria will react identically with no genetic advantage or disadvantage.

The frequency of clinical mono-resistance identified by Pablos-Mendez et al. (1998) and the mutation rate to individual anti-tubercular agents is given in table 3.1. There is not a direct correlation between mutation rate and frequency of clinical isolation. Variation in antibiotic usage may explain the lack of correlation of clinical mono-resistance and mutation rate, or the mutation rate may not be the rate-determining factor in clinical mono resistance.

Ramaswamy & Musser (1998), in a review of antimicrobial resistance in mycobacteria, compiled a list of the various mutations identified in the hot spot of the *rpo*B gene and the number of clinical isolates reported. The mutation position and the number of

clinical isolates identified with that mutation are illustrated in figure 1.2. It is apparent that some *rpo*B mutants are isolated more frequently than others are. The mutation at position 531 in the *rpo*B gene Ser531 to Leu occurred in 237 (49%) out of 478 isolates.

Table 3.1 Median frequency of mono resistance reported world-wide (Pablos-Mendez et al. 1998) and mutation rate of antibiotic resistance of *M. tuberculosis* (David 1970)

	Median frequency of clinical mono-resistance identified world wide (Pablos-Mendez et al. 1998)	Mutation rate to resistance measured in *M. tuberculosis* (David 1970)
Streptomycin	2.5 %	2.56×10^{-8}
Isoniazid	3.2%	2.95×10^{-8}
Rifampicin	0.2 %	2.25×10^{-10}
Ethambutol	0.8%	1.0×10^{-7}

The hypothesis tested in this chapter is that alleles of rifampicin resistance identified by Ramaswamy & Musser (1998) as the most frequently clinically isolated, are formed at a higher mutation rate. This assumes that the mutation rate is the limiting factor, the infecting strain of *M. tuberculosis* must adopt any available rifampicin resistant mutant under rifampicin antibiotic selective pressure. This leads to evolutionary drift. Those mutants most likely to occur will be selected more frequently than those mutants less

likely to occur. The mutation rate is proportional to the rate of isolation; those mutants isolated most frequently having a higher mutation rate than those mutants occurring less frequently.

3.2 Method

3.2.1 Isolation of rifampicin resistant mutants

A flow diagram describing the method used to isolate and identify the *rpo*B mutations leading to rifampicin resistance is given in figure 3.1. *M. tuberculosis* H37Rv was cultured in eight 100-ml Middlebrook 7H9 broths (Difco, Hemel Hempstead, United Kingdom) supplemented with ADC enrichment (Difco, Hemel Hempstead, United Kingdom) for 3 to 4 weeks at 37°C. The inocula used for each of these broths was 100 ul taken from a 2 week 7H9 broth of *M. tuberculosis*. The broth culture was concentrated by centrifugation at 2,000 x g for 30 minutes, and the deposit volume measured. A Miles and Misra viable-cell count was performed on 0.1-ml of the cell deposit to estimate the total number of cells (See Method 2.5). The remaining deposit was spread on the surface of a series of Middlebrook 7H10 agar plates (Difco, Hemel Hempstead, United Kingdom) supplemented with OADC enrichment (Difco, Hemel Hempstead, United Kingdom) containing 5 or 10 mg per litre of rifampicin (Sigma, Poole, United Kingdom). The plates were sealed in polyethylene specimen bags to prevent desiccation and incubated at 37°C for 4 weeks (see figure 3.1).

The total number of colonies with dispersed growth on the plates was counted, and the total number of resistant cells was estimated as colonies counted /proportion of broth deposit plated. The median number of resistant cells formed by spontaneous mutation in the eight broths (T_m) was estimated and the number of mutation events (M_e) occurring in the broths was calculated using the formula devised by Crane et al. (1996):

Formula 3.1 $M_e = (T_m-0.693)/(\ln T_m+0.367)$.

Where M_e is the number of mutation events that have occurred in the median broth and T_m is the median number of mutant colony forming units in the series of broths.

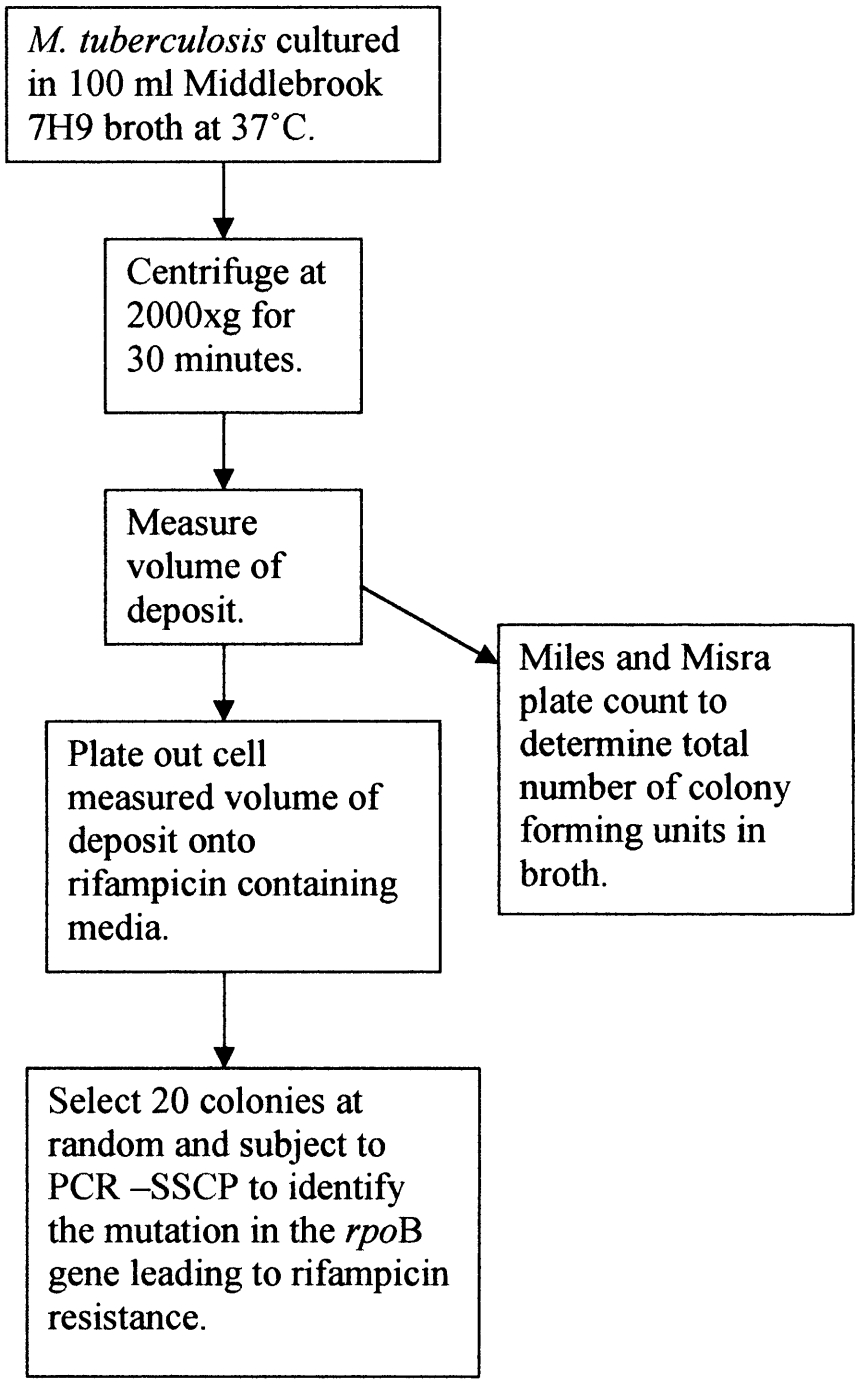
This was used to estimate the mutation rate (μ) by using the median broth viable cell counts as follows $\mu = M_e$/ median broth total colony forming units.

The mutation rate for individual alleles was estimated by first calculating the proportion of each allele present in each broth. This was estimated by subjecting approximately 20 colonies from each experiment to PCR-SSCP (see chapter 2.3). The proportion of this sample giving each SSCP pattern was then multiplied by the total number of resistant cells estimated. The median number of each mutant type was used to calculate the number of mutation events and so the mutation rate using the formula of (Crane et al. 1996).

3.2.2 Selection of rifampicin resistant mutants for further study

Samples of approximately 20 colonies were subcultured from each broth that was used to isolate rifampicin-resistant mutants. The colonies were selected by drawing a line across a plate and picking all the colonies on that line. Each colony was confirmed as rifampicin resistant by culture in a Middlebrook 7H9 broth containing 5 mg/L of rifampicin. DNA was extracted from the isolated colony and subject to PCR-SSCP as described in chapter 2.3. The *rpo*B gene from colonies showing different PCR-SSCP patterns were sequenced as described in chapter 2.6.

Figure 3.1 Outline of the protocol used to isolate and identify rifampicin resistant mutants.



3.3 Results

M. tuberculosis was cultured in eight 100-ml Middlebrook 7H9 broths for 3 to 4 weeks. The first six of the eight broths were plated on both 5mg/L and 10mg/L rifampicin-containing plates. The mean frequency of resistant cells detected was 1.03×10^{-8} on 5 ug/ml plates and 9.87×10^{-9} per cell grown 10 μg/ml plates (see table 3.2). This difference was not significant (P= 0.57) with the matched pair t test. A parametric statistical test was used here to test if the broths contain more bacteria capable of growth at 5 than at 10 ug/ml rifampicin. A paired t test means that each broth is matched with itself and is not compared with other broths in the series. Analysis of the results of these six broths indicated that there was no difference in the frequency of mutants isolated at 5 and 10 μg/ml and so only 5 μg/ml was used on the last two broths.

Figure 3.2 Mutation detection enhancement gel electrophoresis demonstrating representative resistant genotype SSCP pattern A, B and C and the sensitive genotype H37Rv.

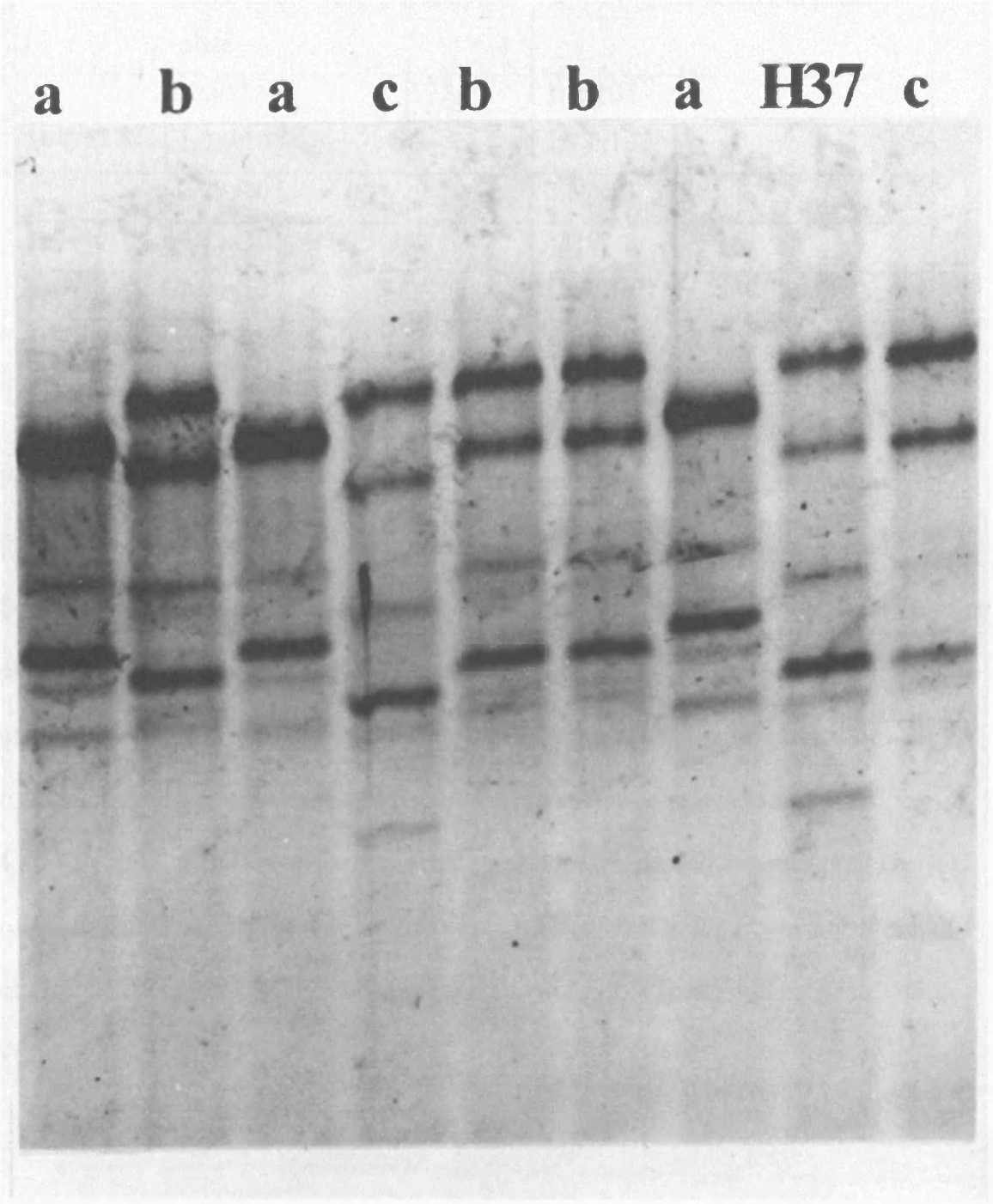


Table 3.2 Rate of mutants isolated from six broths plated out on 5 and 10 mg/L rifampicin. The rate is measured as mutant cells per cell plated. This measurement overcomes the effect of different volumes being plated on different media.

Broth	Rate of mutants per cell isolated on 5-mg/L rifampicin plate.	Rate of mutants per cell isolated on 10-mg/L rifampicin plate.
A	$2x10^{-8}$	$1.5x10^{-8}$
B	$1.05x10^{-8}$	$1.28x10^{-8}$
C	$2.79x10^{-9}$	$2.86x10^{-9}$
D	$4.35x10^{-9}$	$5x10^{-9}$
E	$2.06x10^{-8}$	$1.99x10^{-8}$
F	$3.64x10^{-9}$	$3.66x10^{-9}$

PCR-SSCP analysis of 20 colonies selected at random from each broth revealed only three PCR-SSCP patterns from the *rpo*B gene. These patterns were designated type A, B and C and are illustrated in figure 3.2. The estimated numbers of each pattern present in each of the eight broths is given in table 3.3. The median number of mutants was used to estimate the mutation rate for each mutant type. The total mutation rate for all rifampicin resistant mutations was 6×10^{-10}. The mutation rates were 2×10^{-10}, 4×10^{-10}, and 2×10^{-10} mutations per cell per generation for SSCP patterns A, B and C respectively. No significant difference could be identified in the frequency of mutants of PCR-SSCP pattern A, B and C indicating no difference in the mutation rate (Mann-Whitney U test, $P>0.5$).

A selection of the mutants from each PCR-SSCP pattern was sequenced. The mutants were selected so that mutants with the same PCR-SSCP pattern came from separate broth cultures so that the mutations had arisen independently for each isolate (table 3.4).

Table 3.3 Number of mutants present in broths, after correction for proportion of broth plated and proportion of resistant cells subject to PCR-SSCP. The row marked ^ was from a broth in which the cell count could not be made due to a fungal contaminant

Total mutations rifampicin resistant	SSCP pattern A	SSCP pattern B	SSCP pattern C	CFU in broth
118	21	76	11	1×10^{10}
96	5	62	29	3×10^{10}
124	12	107	6	3×10^{10}
219	41	133	41	8×10^{11}
72	23	19	33	6×10^{11}
1220	933	311	156	4×10^{10}
444	313	67	67	8×10^{11}
	22	93	29	^

Table 3.4 The PCR SSCP pattern and sequence alteration identified in the *rpo*B gene.

PCR-SSCP pattern A	No. of isolates	PCR-SSCP pattern B	No. of isolates	PCR-SSCP pattern C	Number of isolates
His 526⇒ Tyr CAC ⇒ TAC	2	Ser 531⇒ Leu TCG ⇒ TTG	3	His 526⇒ Arg CAC ⇒ CGC	3
His 526⇒ Asp CAC ⇒ GAC	1				

3.4 Discussion

The number of colonies growing on 5 and 10 μg/ml rifampicin was compared from the same broth. This turned this part of the experiment into a simple comparison of the frequency of cells growing in the same or matched broth. Statistical theory states that cell counts should obey the normal distribution and enables the colonies capable of growth on 5 and 10 ug/ml rifampicin to be compared using statistics based on the normal distribution (Meynell & Meynell 1970). The number of mutants growing in a broth does not obey a normal distribution. The comparison of different mutation frequencies could not then be based on the normal distribution. It is for this reason the Mann-Whitney U test has been used to compare the frequency of mutants in different broths (Rosche & Foster 2000).

The hypothesis, that more frequent clinically isolated *rpo*B mutations are associated with a higher mutation rate, must be rejected. The Mann-Whitney U test ($P>0.05$) used to compare the frequency of mutants of different PCR-SSCP types indicates no differential accumulation of mutant types.

The range of SSCP types identified in this study was very much smaller than that identified from clinical samples. Moghazeh et al. (1996), Bodmer et al. (1995) and Ohno et al. (1996) compared the MIC and *rpo*B mutation in a number of clinical isolates (table 3.5). Three mutant types Ser 531⇒ Leu, His 526⇒ Asp, and Ser 531⇒ Trp were associated in all three studies with an MIC of > 8 mg/L rifampicin. In this study only the first two were isolated *in vitro* by rifampicin selection.

Variation in the MIC occurs with different methods used to measure the MIC. Tests on LJ media produce higher MIC values than tests on Middlebrook media, where the MIC can be reduced by the addition of tween. Tween makes the cell more permeable to rifampicin and so increases the intracellular rifampicin concentration (Hui et al 1977). Moghazeh et al. (1996) and Bodmer et al. (1995) used the proportional technique, Moghazeh et al. (1996) using Middlebrook 7H10 and Bodmer et al. (1995) using the Bactec method with Middlebrook 7H12 broth. Ohno et al. (1996) used an absolute MIC technique using 7H9 broth. Tween may have been present in the broth used by Ohno et al. (1996) its presence or absence not being noted. The variation in technique may have been responsible for some of the variation in MIC between the studies. Ohno et al. (1996) finding in general the mutations associated with lower MIC values.

In this study the presence of tween in the culture broth preparing cells for selection may have influenced the selection of mutants. The cell wall being more permeable to rifampicin when it is plated out on rifampicin containing media. The high concentration of rifampicin used may also have excluded mutants that are clinically rifampicin resistant, but are selected at lower effective rifampicin concentrations *in vivo*.

The mutation rate does not appear to be a limiting factor for the appearance of rifampicin resistant mutants *in vivo*. Morlock, Plikaytis & Crawford (2000) using the Po technique identified a higher mutation rate for the most common mutant type Ser 531⇒ Leu relative to the other mutant types. Morlock et al. (2000) identified the *rpo*B mutation from a single colony from each broth and as such, may have not identified correctly those broths with more than one mutant type. This question will be more fully addressed in chapter 4.

The ability of a population to evolve is affected by the amount of genetic variation present. The effective population size is related to the chance of selecting two bacteria at random and them both having an identical genome (Brown & Richman 1997). The larger the amount of genetic variation the larger the effective population size (Ne) and the lower the probability of selecting two bacteria at random that are genetically identical.

Table 3.5 Correlation of rifampicin minimum inhibitory concentration with *rpo*B mutation from three studies with clinical isolates of *M. tuberculosis* (Moghazeh et al. 1996, Bodmer et al.1995, and Ohno et al.1996).

Mutation	Moghazeh et al. (1995)	Ohno et al. (1996)	Bodmer et al. (1996)
Pro 511			>8
Lys 513	>32		
Leu 513	>32		>8
Val 515		0.063	
Tyr 516		128	2
Val 516		4	>8
Leu 521		0.063	
Leu 522			>8
Gly 526		8	
Asn 526	16		
Leu 526	8	16	
Arg 526		512	8
Asp 526	>32	>512	>8
Pro 526		512	>8
Tyr 526	>32		>8
Leu 531	>32	512: 128: 64	>8
Trp 531	>32	>512	>8
Tyr 531			>8
Pro 533	>32	1 : 0.5	
Wild type	1	Not stated	0.25 –0.5

A population with a high genetic variation will be more likely to have favourable mutants present under changing environments than a population with a small amount of genetic variation. This in turn means a population with a high genetic variance is more likely to be able to adapt to a changing environment. This will not just affect evolution to antibiotics, but adaptation to other local host conditions. If the mutation rate to rifampicin resistance, approximately 10^{-10} per bacteria per generation, is not a limiting factor for evolution in *M. tuberculosis*, then this argues for a high value for genetic variation in the host.

Periods of antibiotic therapy followed by lack of effective therapy, as discussed by Mitchison (1998) are associated with emergence of drug resistance. This periodic therapy leads to an effective higher growth rate of the drug resistant mutants relative to the susceptible parental cell line. Multi-drug resistance then emerges when this growing pool of mono-resistant cells generates a lineage containing two drug resistant mutations.

Genetic variation in a population is increased by mutation. This increase in variation needs to be controlled or the cells suffer information death. The constant accumulation of mutations will lead to the information coded in the genome being lost. Stabilising selective pressures will exclude those unfavourable mutants so restricting the accumulation of genetic variation (Bell 1997).

The mutation rate calculated in this study for a single base change was 2×10^{-10} per bacterium per generation. The genome of *M. tuberculosis* consists of 4,411,529 base

pairs (Cole et al. 1998). This gives a probability of a base change at each generation of 0.000882 per bacteria. This compares with the calculations of Drake (1991b) who identified a general mutation rate for all DNA based organisms of 0.0033 mutations per genome per DNA replication. The mutation rate per base at the *rpo*B gene appears slightly lower than the universal mutation rate calculated by Drake (1991b). The *rpo*B gene is however an essential gene and has a lower mutation rate (Koch 1981). The mutation rate of other genes would be needed to be compared to determine if *M. tuberculosis* has a higher or lower mutation rate than is found in other bacteria.

In this chapter I have demonstrated that there is no significant difference in the mutation rate for different alleles of rifampicin resistance. The rate of mutation can not be used to explain the differential rate of isolation of rifampicin resistant alleles described by Ramaswamy & Musser (1998). In the next chapter the growth rate of *M. tuberculosis* with different *rpo*B mutations was compared to determine if differential reproduction could explain the distribution of rifampicin resistance alleles isolated clinically.

Chapter 4

The fitness of *rpo*B mutations

4.1 Introduction

As outlined in the introduction to chapter 3 the alleles of *rpo*B leading to rifampicin resistance are found with different frequencies from clinical cases of MDR-TB. In chapter 3 the hypothesis that these different clinical frequencies reflected a difference in the mutation rate and that some *rpo*B mutations occur more frequently than others was tested. No evidence for this difference in the mutation rate was identified. In this chapter the hypothesis is tested, that a difference in growth rate enables some bacteria with *rpo*B mutations to out compete other bacteria which bare *rpoB* alleles for rifampicin resistance which lead to retarded growth. The hypothesis is that evolution of rifampicin resistance is determined by Darwinian survival of the fittest allele.

The *rpo*B gene encodes the β-protein of RNA polymerase. Von Hippel, Bear, Morgan et al. (1984) described RNA polymerase is a key enzyme in the economy of the cell. Correct functioning of RNA polymerase ensures that not only are the appropriate genes transcribed, but that transcription occurs at the right time and in the required amounts. This requires the enzyme to be responsive to a number of controlling factors.

The β protein of RNA polymerase has a role in the stability of the DNA-RNA polymerase interaction. Mutation in the *rpo*B gene leading to resistance to rifampicin in

E. coli has been described as reducing the stability of RNA polymerase DNA interactions (Zhou & Jin 1998). Stringently controlled promoter sites with very weak RNA polymerase-DNA binding were further weakened by these *rpo*B mutations, leading to loss of gene expression. This indicates that mutations in the *rpo*B gene can affect the repertoire of genes recognised.

RNA polymerase must not only recognise promoter sites but is also involved with termination of transcription. This requires recognition of the termination sequence and can involve other proteins. Point mutations in the *rpo*B gene have been described in *E. coli* that produce phenotypes with altered transcription termination characteristics (Jin & Gross 1988).

Rifampicin resistance in *E. coli* has been associated with a variety of phenotypic side effects, including temperature sensitivity, and auxotrophy for glutamine (Wehrli 1983). The role of RNA polymerase in the cell means that mutations in the *rpo*B region can have a direct effect on the transcription of other genes. This in turn can affect the ability of the cell to produce proteins and enzymes and so the *rpo*B allele can affect the cell's ability to reproduce

Mayr (1997) briefly outlined two forms of selection, which he names survival and reproductive. Reproductive selection achieves its effect by differential growth rates of cells. Reproductive selection can only operate if the competing organisms are both present and able to survive and reproduce. Tempest (1978) produced a formula

(formula 4.1) which enables the proportions of two competing cell types to be calculated. In this chapter fitness is defined by Tempest's model. The competitive ability of an organism is calculated by the number of generations of each type of organism produced in a mixed broth.

Formula 4.1 $Na/Nb = 2^{(ga-gb)}.N^a/N^b$

Where Na/Nb = The ration of cells a and b after a time interval

ga and gb = The number of generations of organisms a and b respectively that occur in that time interval

N^a/N^b = The ratio of cell types a and b respectively at time zero

Bell (1997) describes mutation in a haploid cell line as generating its own cell lineage. The different cell lineages then must compete, if the different alleles reproduce at different rates then that competition will be by reproductive selection. Differential rates of reproduction generate a difference in allele frequency leading to the predominance of one allele type as described by formula 4.1.

Tempest's model of competition allows a small reproductive difference to produce a large change in the frequency of alleles. Small differences in fitness will, given time, alter the frequency of alleles. An organism with an assumed fitness of 0.99 composing 99% of the population, in competition with an organism with a fitness of 1.0 occupying the remaining 1% of the population, declines in frequency to below 0.5 after

approximately 13 generations. The smaller the difference in fitness between alleles, the longer it takes for the fitter allele to come to predominate.

In this chapter the ability of clones with known *rpo*B mutations to reproduce and compete with the rifampicin susceptible parent clone was measured. The hypothesis was that the frequency of clinical isolation of the rifampicin resistant allele would be reflected in the relative fitness of that *rpo*B allele in competition with the parent rifampicin susceptible *rpo*B allele.

4.2 Method

4.2.1 Bacteria

Rifampicin resistant mutants isolated from the parent fully susceptible strain (H37Rv) were isolated as described in chapter 3. Three colonies from each of the SSCP patterns described in chapter 3 were sequenced. The isolation of rifampicin resistant mutants involved growing a broth culture of *M. tuberculosis* H37Rv and plating the entire culture onto media containing 5 and 10 μg/ml rifampicin. The rifampicin resistant clones with each SSCP pattern originated from a single colony picked from a separate broth to reduce the possibility that mutants with the same SSCP pattern had a more recent ancestor than the rifampicin susceptible parent strain. The clones used had the *rpo*B region associated with rifampicin resistance tested using the SSCP technique and sequenced as described in chapter 3.

4.2.2 Fitness assay

A flow diagram describing the method used to determine the fitness of the *rpo*B mutations leading to rifampicin resistance is given in figure 4.1. Broth cultures of *M. tuberculosis* H37Rv fully sensitive (the parent strain) and each of the rifampicin resistant clones was grown to approximately 0.5 McFarland over approximately 2 to 3 weeks in a 4-ml Middlebrook 7H9 broth culture. The Miles and Misra plate count technique was used to estimate the viable cell count as described in section 2.7. This estimates the number of rifampicin resistant bacteria present by counting the colony forming units formed on media containing rifampicin. The total number of organisms was estimated by counting the number of colony forming units growing on media with no antibiotic present, that is both the rifampicin resistant and susceptible bacteria. By subtracting the estimate of the number of rifampicin resistant organisms from the total number, the number of susceptible organisms can be estimated. As explained in section 2.8 the estimate of the susceptible organisms is more accurate if a low number of rifampicin resistant organisms are present. This was achieved by using a lower inocula of rifampicin resistant cells than susceptible cells. A 10 fold dilution of rifampicin susceptible and 100 fold dilution of rifampicin resistant cells were prepared; and from each a 50µl sample was inoculated into 4 ml of fresh Middlebrook 7H9 broth to create a mixed culture of rifampicin resistant and susceptible cells.

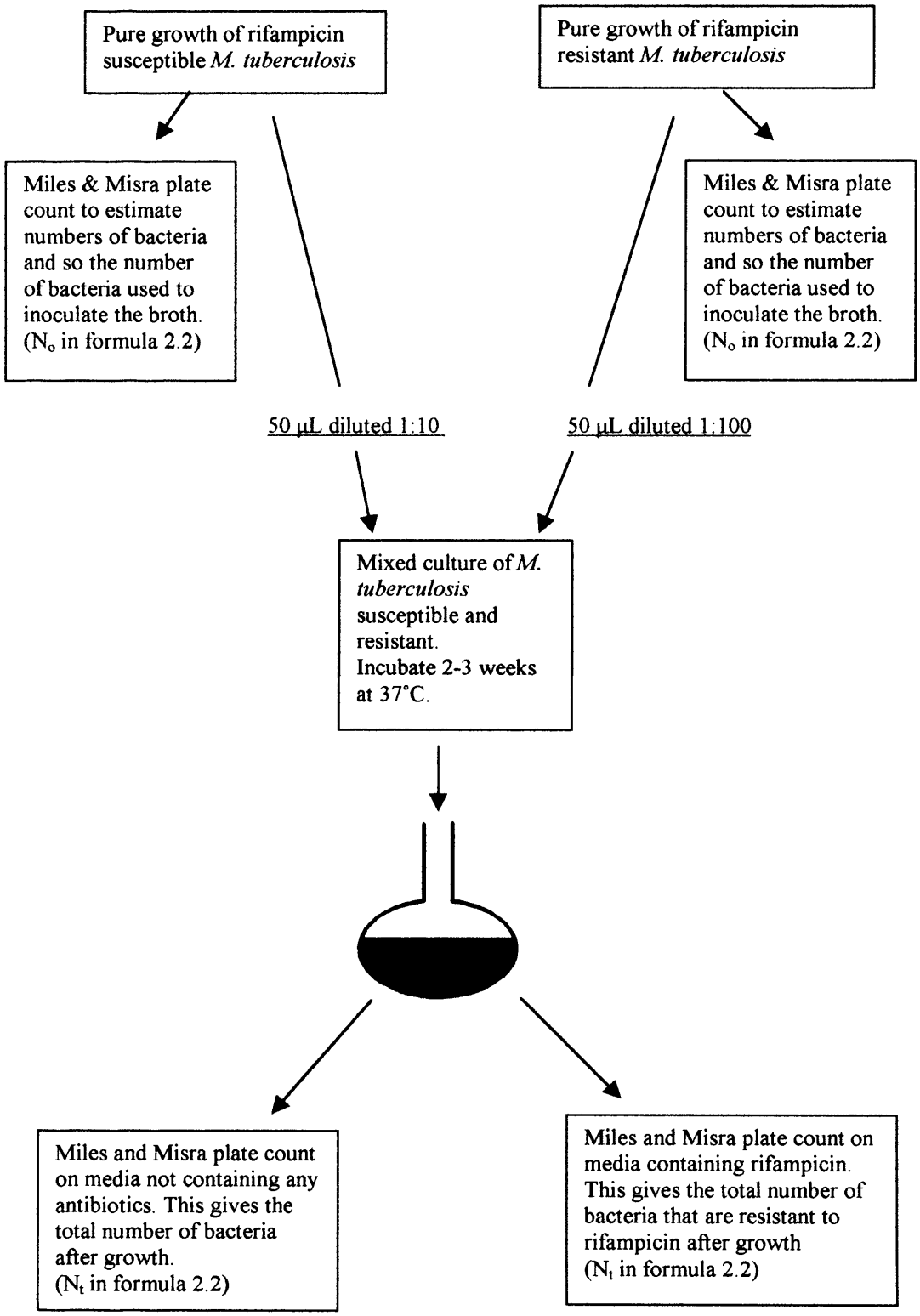
After 2 to 3 weeks of incubation at 37°C when growth had reached about 1 McFarland, a count of viable cells was performed by the Miles and Misra technique on media containing 5 mg/L rifampicin and drug free media as described in section 2.7. The

number of rifampicin resistant cells present was estimated from the number of colonies growing on the rifampicin containing media. The number of colonies growing on the drug free media was used to estimate the total number of cells present, both rifampicin resistant and susceptible in the mixed broth culture. The number of rifampicin susceptible cells present could then be calculated by subtracting the numbers of rifampicin resistant cells from the total number of cells (see section 2.8.). The number of generations of rifampicin resistant and rifampicin susceptible bacilli growing in the mixed broth culture could then be calculated as given in section 2.8. The fitness of the mutant cell (rifampicin resistant) line is the ratio of generations formed compared to the parent (susceptible) cell line.

4.2.3 Statistical methods

Regression analysis was performed with Unistat version 1.13 and JMP IN version 3.17 (SAS Institute Inc) to identify if the frequency of isolation clinically was reflected in the fitness associated with the presence of the three *rpo*B mutations examined. The difference in generations of rifampicin resistant and susceptible clones were compared using students t test using Excel version 4.0 and JMP IN version 3.17 (SAS Institute Inc) to confirm higher growth rates by the susceptible parent strain.

Figure 4.1 Flow diagram of the relative fitness experiment comparing the numbers of generations formed in mixed culture of both rifampicin susceptible (parent) cells and rifampicin resistant (mutant cells).



4.3 Results

Clones 26, 35 and 112 had a *rpo*B PCR-SSCP pattern type A. The *rpo*B gene was sequenced and mutation at codon 526 was identified. These mutations were clones 26 and 112 (His 526 Tyr) and clone 35 (His 526 Asp). Ramaswamy & Musser (1998) reported 19% of clinical rifampicin resistant isolates containing the mutation His 526 Tyr in the *rpo*B gene, whilst His 526 Asp was responsible for 6% of rifampicin resistant isolates.

Clone 7, 68, 88 and 209 had a *rpo*B PCR-SSCP pattern type B. The *rpo*B gene was sequenced and mutation at codon 531 in the *rpo*B gene (Ser 531 Leu) was identified in all clones. This mutation was reported by Ramaswamy & Musser (1998) to be responsible for 49% of clinical rifampicin resistant isolates.

Clones 66, 118 and 25 had an *rpo*B PCR-SSCP pattern type C. The *rpo*B gene was sequenced and mutation at codon 526 in the *rpo*B gene (His 531 Arg) was identified in all clones. This mutation was reported by (Ramaswamy & Musser 1998) to be responsible for 4% of clinical rifampicin resistant isolates.

Tables 4.1 to 4.3 show the relative fitness of the rifampicin resistant clones compared to the fully susceptible parent clone. Clones with SSCP pattern A and C do not form as many generations as do their susceptible parents ($P =< 0.02$; students paired t test). Four clones with SSCP pattern B had their fitness determined relative to the susceptible

parent (table 4.2). There was no significant difference between mutant type B and its susceptible parent (p=0.09). Mutations associated with PCR-SSCP patterns A and C suffered a reduction in fitness relative to the susceptible parent clone (see tables 4.1 and 4.3). Using data on the clinical frequency of resistance mutations presented by the review of Ramaswamy & Musser (1998), it was possible to correlate the frequency of clinical isolation and the *in vitro* fitness of different mutations as determined in these experiments (see figure 4.2 regression analysis p=0.026). There is a direct correlation between the frequency of clinical isolation of *rpo*B mutation and the *in vitro* relative fitness associated with the *rpo*B mutation.

Table 4.1 Relative fitness of SSCP pattern A mutants.

	NUMBER OF GENERATIONS				MEAN FITNESS	MUTATION
strain 26	8.1	7.1	5.7	6.9	0.80	CAC 526 ⇒TAC
H37Rv	9.6	8.6	8.4	8		
strain 35	2.8	3.0	2.6		0.42	CAC 526⇒GAC
H37Rv	5.4	7.6	7.2			
strain 112	4.2	4.9	4.9	4.5	0.79	CAC 526⇒TAC
H37Rv	5.4	6.5	5.4	6.3		

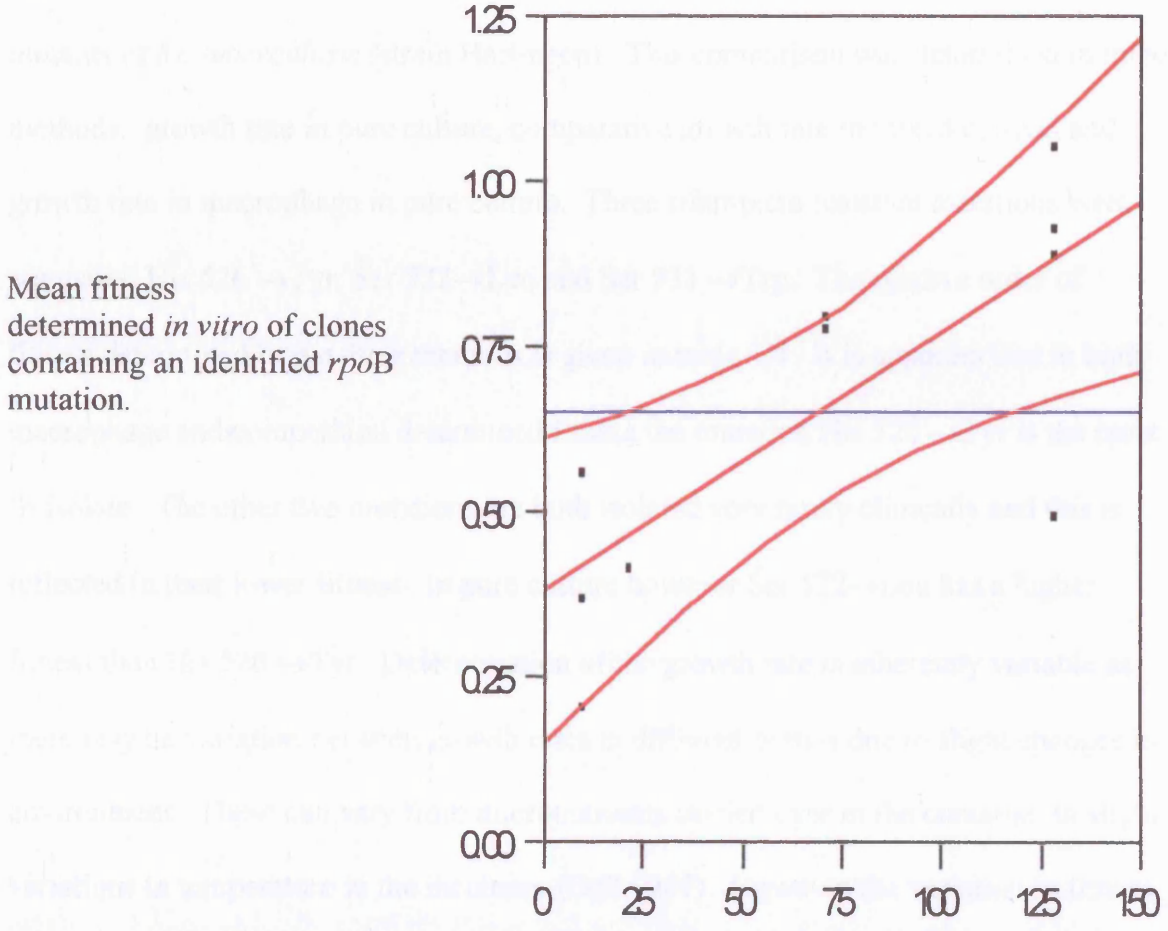
Table 4.2 Relative fitness of SSCP pattern B mutants.

	NUMBER OF GENERATIONS					MEAN FITNESS	MUTATION
strain 7	9.3	4.7	4.9			0.90	TCG 531⇒TTG
H37Rv	8.6	5.7	6.2				
strain 68	5.4	4.8	4.3			0.50	TCG 531⇒TTG
H37Rv	10	9.6	9.2				
strain 88	7	11	4.3			0.93	TCG 531⇒TTG
H37Rv	7.7	10.3	5.2				
strain 209	8.1	9.2	8.8	7.6	7.8	1.1	TCG 531⇒TTG
H37Rv	8.3	9.2	8.5	6.6	6.9		

Table 4.3 Relative fitness of SSCP pattern C mutants.

	NUMBER OF GENERATIONS					MEAN FITNESS	MUTATION
strain 66	1.1	1.6				0.21	CAC 526⇒CGC
H37Rv	6.3	6.5					
strain 118	3.6	1.4				0.38	CAC 526 ⇒CGC
H37Rv	7.3	5.4					
strain 25	4.8	4.7	5.6	6.3	7.0	0.57	CAC 526⇒CGC
H37Rv	10.4	9.4	10.5	10.2	9.7		

Figure 4.2 Correlation of mean fitness of *rpo*B mutations identified *in vitro* and the number of clinical isolates reported by Musser (1995) in a review of reported clinical isolates. The four most commonly clinically isolated *rpo*B mutations leading to rifampicin resistance had the fitness estimated Ser 531 Leu (128 reported isolates), His 526 Tyr (70 reported isolates), His 526 Asp (21 reported isolates), and His 531 Arg (9 reported isolates). The graph shows the upper and lower 95% confidence limits of correlation (plotted using JMP IN version 3.17 (SAS Institute Inc)).



4.4 Discussion

4.4.1 The correlation between isolation rate and fitness in the *rpo*B gene

There is a positive correlation (p=0.026) between the rates at which mutants in the *rpo*B gene causing rifampicin resistance are reported and the relative fitness of *rpo*B mutant clones measured *in vitro* (figure 4.2). This indicates that the relative fitness of the *rpo*B mutation affects the probability that it will rise to predominance clinically.

Mariam, Mengistu, Hoffner et al (2004) compared the growth rate of rifampicin resistant mutants of *M. tuberculosis* (strain Harlingen). This comparison was determined in three methods: growth rate in pure culture, comparative growth rate in mixed culture, and growth rate in macrophage in pure culture. Three rifampicin resistant mutations were compared His 526 →Tyr, Ser 522→Leu and Ser 531 →Trp. The relative order of fitness determined in the three methods is given in table 4.4. It is apparent that in both macrophage and competition determined fitness the mutation His 526 →Tyr is the most fit isolate. The other two mutations are both isolated very rarely clinically and this is reflected in their lower fitness. In pure culture however Ser 522→Leu has a higher fitness than His 526 →Tyr. Determination of the growth rate is inherently variable as there may be variation between growth rates in different bottles due to slight changes in environment. These can vary from micronutrients carried over in the container to slight variations in temperature in the incubator (Bell 1997). However the variation in fitness values does indicate that caution is required in interpreting fitness values determined *in vitro* and their comparison to the situation *in vivo*.

Table 4.4 Fitness of rifampicin resistant alleles of *M. tuberculosis* (strain Harlingen) determined by Mariam et al. (2004) using three methods. Growth rate in pure culture in Middlebrook 7H9 broth, growth rate in mixed culture, and growth rate in pure culture in Macrophage.

MUTATION	FITNESS DETERMINED BY GROWTH RATE IN PURE CULTURE	COMPETITION DETERMINED RELATIVE FITNESS	PURE GROWTH IN MACROPHAGE	CLINICAL ISOLATION % BASED ON RAMASWAMY & MUSSER (1998)
His 526 →Tyr	0.86 ± 0.03	0.89 ±0.05	0.63 ± 0.02	20 %
Ser 522→Leu	0.95 ± 0.02	0.54 ± 0.03	0.50 ± 0.16	1 %
Ser 531 →Trp	0.71 ± 0.09	0.67 ± 0.06	0.28 ± 0.06	1 %

Morlock et al (2000) identified a higher frequency of the most frequent clinical *rpo*B mutant type (Ser 531 to Leu) and concluded the raised mutation rate of Ser 531 to Leu was the reason for this mutant types high rate of clinical isolation. In chapter 3 a raised mutation rate for this resistance allele was not detected.

Morlock et al (2000) identified a far higher range of mutant types than was identified in this study (table 4.5). A possible explanation for this is that a lower concentration of rifampicin (1ug/ml) was used by Morlock et al (2000). than was used in this study (5 ug/ml) to isolate rifampicin resistant mutants. Comparison of the MIC to rifampicin of clinical isolates and the *rpo*B mutation has shown some variation in the MIC both for different alleles of *rpo*B and with different isolates with the same *rpo*B mutation (see table 3.6). The variation in rifampicin MIC for isolates with the same *rpo*B mutation reflects the situation in coliforms. Wehrli (1983) found no direct correlation between

the MIC of coliforms and the concentration required to inhibit by 50% RNA production by purified RNA polymerase from rifampicin resistant isolates. The lack of correlation indicates in coliforms the MIC is affected by other factors such as cell permeability in addition to the *rpo*B allele.

Morlock (2000) adopted the Po technique as described by (Luria & Delbrück 1943) to compare the mutation rate of *rpo*B alleles. A total of one hundred 5-ml Middlebrook 7H9 broth cultures of *M. tuberculosis* H37Rv were prepared and incubated with shaking for 32 days at 37°C. The cultures were harvested after incubation. Morlock et al. (2000) describes the technique as: 'Approximately 2 ml of culture containing most of the cell mass was pipetted from the bottom of each tube and transferred to a sterile screw-cap microcentrifuge tube'. The tube was centrifuged and supernatant discarded. The deposit was examined for rifampicin resistant mutations by plating out the deposit on Middlebrook 7H10 agar containing 1 μg/ml of rifampicin. Of the 100 cultures 32 did not have mutations present. The number of mutant colonies isolated per broth varied from 1 to 288 with 62 plates having between 1 and 11 colonies. A single rifampicin resistant mutant was sequenced from each broth except in two broths from which high numbers of mutant colonies were isolated. Morlock et al. (2000) 'expected this procedure to produce independent mutational events in each culture' allowing comparison of the frequency of broths containing different *rpo*B mutations.

Table 4.5 The mutations isolated by Morlock, Plikaytis & Crawford (2000) and the mutations isolated in this study. The frequency of *rpo*B alleles identified by Morlock et al (2000) in all broths and from the group of broths producing 1 mutant colony are shown.

Mutation	Rifampicin resistant mutant frequency reported by Morlock, Plikaytis & Crawford (2000)		This study	Clinical frequency reported by Ramaswamy & Musser (1998)
	All broths	Broths with 1 mutant		
Ser 531 Leu	39 (60.9)	18	PCR-SSCP type B	237
His 526 Tyr	6 (9.4)	2	PCR-SSCP type A	94
His 256 Arg	5 (7.8)	3	PCR-SSCP type C	19
His 526 Asp	4 (6.2)	1	PCR-SSCP type A	31
Ser 521 Leu	4 (6.2)		Not found	7
Ser 531 Trp	2 (3.1)	2	Not found	7
His 526 Pro	1 (1.6)		Not found	6
Asp 516 Val	1 (1.6)	1	Not found	6
Deletion codon 516 GAC	1 (1.6)	1	Not found	
Insertion TTC after codon 513	1 (1.6)	1	Not found	

The Po technique is dependent on the number of broths in which mutants have not arisen. The number of mutation events that have occurred in a broth containing mutants is not known. The number of mutation events that have occurred in a broth with no mutants must be nil. The important characteristic of this technique is that broths need to be scored simply for mutants present or absent. Morlock et al. (2000) assumed each broth will have contained a single mutation event. However, does each broth contain a single mutational event and can this be tested?

Identifying the number of mutational events occurring in a broth is analogous to attempts to isolate pure cultures from broth culture prior to the use of agar plates. This was achieved using the Poisson distribution, the same distribution as is expected in mutation events, and the bases of the Po technique. Morlock et al. (2000) calculated that there was an average of 1.02 mutation events per broth. The generalised Poisson distribution is given in formula 4.2

Formula 4.2 $P = e^{-n} . n^r/r!$

Where r is the number of mutation events = 0,1,2,3 ...

and n = the mean number of events.

Where r = 0 and n is 1.02 the probability the probability of zero mutants becomes the Po formula and is given in formula 4.3

Formula 4.3 $P_0 = e^{-1.02}$

The probability of a single mutant being present where the average number of mutations n is 1.02 is given by substituting a value of r = 1. This is produced in formula 4.4.

Formula 4.4 $P_1 = 1.02. e^{-1}$

This enables the calculation that the probability that a broth contains a single mutation event $P_1 = 0.368$ and no mutation events $P_0 = 0.36$. The probability a broth contained more than a single mutation event then becomes $P_{>1} = 1- (0.368+0.36) = 0.27$.

If more than one mutation is present then selection of a single colony will skew the results towards those mutants that have achieved greatest numbers. Mutants can be present at higher frequencies either from earlier mutation or faster growth rates of the mutants. In this study, 20 colonies were picked from each broth to estimate mutant frequencies and the frequency of mutants was compared using non-parametric methods. Non-parametric statistics are recommended to compare mutant frequencies. These non-parametric statistical tests do not assume a normal distribution and so are not as affected by the skew in frequency (Rosche & Foster 2000). There is a probability that some of the broths assumed to contain a single mutation event contained more than one mutant in the study conducted by Morlock et al. (2000). Where two or more mutations have occurred the increased probability that more rapid growing mutants will be detected,

may have skewed the frequency of *rpo*B mutants towards detection of more rapidly growing *rpo*B alleles.

There is a very short window of time in the history of the broth, after a mutational event but prior to the mutant cell dividing, called the 'phenotypic lag'. This arises due to the necessity of replacing ancestral gene products with the parent lineages. The cell will not show resistance until resistant *rpo*B gene products replace sensitive *rpo*B gene products. Phenotypic lag has a larger effect on the small number of mutational events used in the Po technique than techniques with more mutational events (Rosche & Foster 2000). The technique used in chapter 3 to determine mutant frequency is not as heavily affected by phenotypic lag due to the large number of mutations that have arisen in each broth. Mutation types with low relative fitness and therefore longer generation times may be expected to suffer a longer phenotypic lag than mutants with higher relative fitness. This difference in phenotypic lag may account for the skew in mutant types identified by Morlock et al. (2000) in the group where a single rifampicin resistant colony was isolated.

Morlock et al. (2000) attempted to exclude the number of broth cultures with more than one mutation event by only using those broths in which a single mutant colony was isolated. From single isolates Morlock et al. (2000) identified a bias of 18 out of 29 (62%) mutants of the type Ser 531 to Leu. Morlock et al. (2000) did not plate out all of the broth and so whether isolation of a single mutant cell indicates a single mutant was present in the broth is uncertain.

The second most common mutation type reported by Ramaswamy & Musser (1998) His 526 to Tyr (see table 3.2) did not have a raised isolation rate if only those broths showing a single mutant colony are considered. This is despite His 526 to Tyr being isolated more than three times more frequently than the next most frequent mutant type. The determination of the relative fitness gives the same order of relative fitness and the clinical isolation rate for the four mutant types identified. This indicates that relative fitness of mutants is important to the rate of clinical isolation.

4.4.2 Will the mutant with the highest relative fitness always be isolated?

Bell (1997) states that 'the sorting limit of asexual populations is the extent of variation'. This means that a selective pressure will not affect a population if there are no available mutants with an enhanced relative fitness under that selective pressure available. This emphasises the importance of the range of mutants in a bacterial population at the start of a lethal selective pressure such as antibiotic treatment.

Small populations such as emerging antibiotic resistant populations are very susceptible to chance events. Schuster & Sigmund (1989) considered the probability of an advantageous mutation coming to predominance in a population. A boundary layer was described below which the population could become extinct despite a selective advantage. Consider a hypothetical organism with an assumed growth at the end of each generation producing 2 cells for every cell and a 40% chance of each cell dying before reproducing. As the probability of dying is lower than the reproduction rate, the

population is expected to grow. But when a single organism is present the probability of the population dying during the first generation before reproducing is 0.4. The probability that the same population will all be eliminated in a single generation when the population becomes greater than 10 cells is very small ($p \leq 10^{-4}$). An antibiotic resistant cell during an infection is prone to just such chance events. The cell may be resistant to the antibiotic therapy, but the immune system can kill that cell prior to infection becoming established or the cell simply shed from the host.

Frost & McLean (1994) studying quasi-species and the development of zidovudine resistance in HIV, argued that initial quasi species distribution prior to drug treatment will have an important effect on the allele that comes to predominate. The sequential emergence of mutations in HIV during treatment was accompanied by a sequential increase in drug resistance. Frost & McLean (1994) stated that: 'The patterns that emerge during therapy are deterministic because mutation does not play an important part in the dynamics compared with competition. However the level of resistant mutants is determined stochastically (by mutation). Variability in the emergence of resistance between individuals can be credited to this pre-therapy stochasticity'.

The allele of rifampicin resistance that emerges in *M. tuberculosis* clinically will have a similar stochastic and deterministic component. Mutation supplying the availability of rifampicin resistant alleles, will be a stochastic determinant. Competition between those mutations, once grown above the boundary where chance can eliminate the population,

will then be deterministic. Those mutations associated with a higher relative fitness will come to predominate.

The range of rifampicin resistant mutations available during infection will be dependant on the infection size and so the probability of a mutant being present. The probability of a rifampicin resistant mutation being present will be dependent on its mutation rate. A population will contain on average one rifampicin resistant mutation if the population size is $1/\mu$ where the rifampicin resistance mutation rate is μ.

Reproductive selection can only operate if more than one allele is present, including the sensitive allele. Following the start of antibiotic therapy rifampicin resistant alleles obtain a selective advantage. If two or more alleles for rifampicin resistance are present these must compete, the allele with the fastest growth rate coming to predominance. If the optimal allele is not present or is lost by genetic drift then it can not be driven by selective pressure to predominance.

The role of chance during tuberculosis infection will ensure that the perfect allele with the highest fitness is not selected on all occasions. In some infections the allele may not be present. In others it may be present in small numbers and lost due to chance events. The higher fitness will however enable it to more frequently come to predominate.

The range of rifampicin resistant mutations available during infection is dependant on the infection size and so is the probability of a mutant being present (Mitchison 1998).

The probability of a rifampicin resistant mutation being present will be dependent on its mutation rate. A population will contain, on average, one rifampicin resistant mutation if the population size is $1/\mu$ where the rifampicin resistance mutation rate is μ. Antibiotic resistant mutants may have a fitness cost relative to the susceptible parent strain in the absence of antibiotic. The proportion of mutants in a continuous culture will be proportional to μ/w, where μ is the mutation rate and w is the selection coefficient (Bell 1997). The smaller the selection coefficient the higher the proportion of the broth culture that can be attained by the less fit clone. The selection coefficient is related to the relative fitness using formula w= 1-Fitness (Bell 1997). The higher the relative fitness of an *rpo*B mutant, relative to the susceptible parent strain, the smaller the selection coefficient and so the higher the proportion of a population that mutant can attain when there is no rifampicin present. If the proportion is high enough and the population large enough then a constant pool of *rpo*B mutants may be available.

The model used by Lipsitch & Levin (1998) to calculate the probability of multi-drug resistant *M. tuberculosis* is based on the mutation rate for resistance to each individual antibiotic. They gave an example, 'if the frequency of drug resistance to drug 1 is 10^{-7} and to drug 2 is 10^{-7}, then on average, a population of 10^{10} bacteria will contain 1000 bacteria resistant to each drug, but the likelihood of observing a bacterium resistant to both drugs will be of the order 10^{-4}'. Lipsitch & Levin (1998) estimate the probability of a bacterium containing a gene for resistance to both antibiotics as 10^{-14}, that is 10^{-7} x 10^{-7}. This is the probability of both mutations producing resistance to drug 1 and drug 2 occurring on the same genome, at the same time. This is based on the theory that

mutation rates control the rate of emergence of resistance. Assuming sequential gene acquisition is more complicated, being dependant on the number of cells with resistance to one of the antibiotics. If selection in part controls the rate of emergence of resistance then the proportion of mutants present during infection will be dependent on the selection coefficient. This alters the probability of a single mutation being present and so a double mutation occurring.

Chapter 5

Comparison of the fitness of two isolates of *Mycobacterium tuberculosis* one of which developed multi-drug resistance during the course of treatment

5.1 Introduction

Anderson (1999) states: 'Drug rotation is essential because it is important to keep varying the selective pressure'. Programs to limit or reduce the rise in antibiotic resistance have been based on the theory that antibiotic resistance has an associated cost to the bacterium.

There is a correlation between the increasing use of an antibiotic and an increase in resistance to that antibiotic. Magee, Pritchard, Fitzgerald et al. (1999) in a study of urinary tract infections found the frequency of resistant coliforms correlates with increasing antibiotic usage by individual general practitioners. The introduction of each anti-tubercular drug has been associated with emergence of resistance to that drug as it is used (Ramaswamy & Musser 1998; Fox, Ellard et al. 1999).

A decline in the frequency of antibiotic resistance on removal of antibiotics has been reported. Sulphonamide resistance in *Neisseria meningitidis* demonstrates an example of such a decline. Sulphonamide was last used for prophylaxis against meningococci in 1986-88. At this point sulphonamide resistance was about 40%, this level of resistance declined to 25% in 1995 (Kaczmarski 1997). Reversion to susceptibility of tuberculosis

has been recorded for streptomycin resistance (Fox, Ellard et al. 1999). However, such reversion to susceptibility does not arise with isoniazid, this despite an apparent reduced virulence reported with isoniazid resistance (see section 1.15).

The reduction in frequency of resistance on removal of antibiotic selective pressure is not universal. Streptomycin has not been used against the Enterobacteriacea for 25 years and yet Chiew, Yeo, Hall et al. (1998) identified over 20% of *E. coli* isolates resistant to streptomycin in their 1998 study. Resistance to streptomycin was associated with transposon Tn21. Transposon Tn21 has the ability to collect antibiotic resistance determining sequences. The ability of Tn21 to accumulate antibiotic resistance has allowed it to play an important role in the spread of antibiotic resistance in gram negative bacteria (Liebert, Hall & Summers 1999). The use of one antibiotic with resistance coded for on Tn21 will help to conserve all of the antibiotic resistance genes held by Tn21.

Retention of antibiotic resistance genes by sharing a genome with antibiotic resistance genes under active selective pressure is not restricted to Tn21. Magee et al. (1999) found that in GP practices, combined resistance to ampicillin and trimethoprim occurred in 21% of isolates. There was a correlation between use of either antibiotic and resistance to both antibiotics. Plasmids with combined resistance to trimethoprim and ampicillin are common in *E. coli* and use of either antibiotic would provide a selective pressure for the retention of these plasmids (Amyes 1989).

Gilliver et al. (1999) studied Enterobacteria isolated from bank voles (*Clethrionomys glareolus*) and wood mice (*Apodemus sylvaticus*) in woodland sites in Northwest England. Gilliver et al. (1999) found 89% of *E. coli* isolates were resistant to amoxicillin and 14% resistant to tetracycline. The presence of antibiotic resistance in a wild animal population indicates antibiotic resistant strains may not be eliminated by removal of antibiotic selective pressure.

The ability of antibiotic resistance genes to be preserved in the absence of antibiotic pressure may be associated with compensatory mutations. Lenski (1988) found that on initial insertion of a plasmid into *E. coli* there was an associated decline in fitness. Continued culture in the presence of antibiotic pressure allowed the *E. coli* strain to adapt to the presence of the plasmid. The fitness cost associated with the plasmid's presence declined. Removal of the plasmid from the clone of *E. coli* adapted to the plasmid's presence revealed a fitness cost with the plasmid's absence. Lenski concluded from this that the genome adapts to its total environment including the environment created by its own genes. Mutation in the genome is an alteration in the cell's environment.

Adaptation to streptomycin resistance by compensatory mutations has been identified in *E. coli* (Schrag, Perrot & Levin 1997). Compensatory mutations in *M. tuberculosis* to isoniazid resistance have similarly been identified (Sherman et al. 1996). However whether there is an associated cost with removal of antibiotic resistance has not been identified.

Bjorkman, Hughes & Andersson (2000) examining compensatory mutations to fusidic acid resistance in *Salmonella typhimurium* strain LT2 found a different compensatory mutation when the bacteria were cultured in mice or Luria Bertani broth (LB) media. Fusidic acid resistance was created by mutation in the *fus*A gene, which codes for elongation factor G. Fusidic acid affects the growth rate when cultured in LB and in mice. Compensatory mutations were selected by serial passage either through LB media (28 isolates), or mice (25 isolates). At each passage the growth rate was determined in LB media and if raised then compensatory mutations were assumed to be present.

Compensatory mutations in mice were predominantly by reversion to susceptibility (14 isolates out of 25). The mutation leading to resistance had been eliminated and the wild type or susceptible form of the gene *fus*A was present. Fusidic acid resistant mutants have alterations in the concentrations of (p)ppGpp which is a regulator of gene expression. Reversion to susceptibility will have returned this regulator to its normal value. Compensatory mutations, while improving the rate of protein synthesis, may not have affected the regulator concentration. Serial passage in LB media almost always led to compensatory mutations, not reversion to susceptibility (26 out of 28 isolates).

There is a single mutation that can lead to reversion to susceptibility. However, there are a large number of possible mutations that can lead to improved gene expression. These compensatory mutations are more likely to arise than true reversion. The failure of compensatory mutations to return the cell regulation to normal may not have affected

bacteria grown on artificial media. However, bacteria grown in mice require full expression of virulence related genes, normal expression of (p)ppGpp may have provided a selective pressure for reversion to susceptibility.

Compensatory mutations that arise *in vitro* are not necessarily the same mutations that arise *in vivo.* Selective pressures operating in these environments will differ, and so the range of adaptive mutations required *in vitro* and *in vivo* are different. The question of whether compensatory mutations to antibiotic resistance carry a cost in the sensitive cell can only be addressed once it is established that resistant mutations carry a selective cost *in vivo.*

Ordway, Sonnenberg, Donahue et al. (1995) examined the ability of *M. tuberculosis* resistant to one or more antibiotics to grow in mice and *in vitro.* 15 isolates of Mycobacteria were compared to the virulent laboratory strain of *M. tuberculosis* Erdman. No correlation between resistance and growth rate was identified, however each of these isolates was unrelated. The genetic variation will have extended beyond antibiotic resistance. To examine the effect of antibiotic resistance it is necessary to examine matched isolates with a common heritage to minimise genetic variation between the isolates.

This chapter is addresses the hypothesis that there is a fitness cost associated with antibiotic resistance in M. tuberculosis *in vivo.* This was studied by comparing the fitness of two isolates of *M. tuberculosis* from matched clinical cases.

5.2 Method

5.2.1 Isolates

Isolates were obtained from siblings who presented at the Royal Free Hospital. The first to present was a male HIV sero-negative (ET). The initial isolate in 1991 was fully sensitive. The infecting strain of tuberculosis developed resistance over a period of 3 years to nine antibiotics (rifampicin, isoniazid, streptomycin, ciprofloxacin, clarithromycin, amikacin, PAS, cycloserine, and capreomycin). No isolates were obtained from ET during the intervening period so it was not possible to state when resistance first developed.

ET's sister (AT) was HIV sero-negative and lived with her brother ET. AT developed tuberculosis 9 months after her brother and this strain remained fully drug susceptible (AT) (Davies et al. 2000a).

The strains of *M. tuberculosis* isolated from both AT (fully drug susceptible) and ET (MDR-TB) were primary clinical isolates. The strains were isolated from sputum samples on Löwenstein Jensen media within 14 days of each other. The laboratory control strain fully drug susceptible H37Rv was used as a control. The original slopes and H37Rv were sub-cultured into Middlebrook 7H9 broth and incubated at 37°C for 21 days. These broths were then used as the inocula for the fitness assay.

5.2.2 PCR sequencing of *rpo*B gene

The technique described in section 2.3 was used.

5.2.3 Assay of relative fitness of drug resistant isolates (ET) compared to reference strain H37Rv

Both drug-resistant (ET) and susceptible strain (H37Rv) were diluted 1:400 into two fresh 4 ml Middlebrook 7H9 broth cultures. These mixed broth cultures were incubated at 37°C for 6 days. At the start and end of this time a Miles and Misra plate count determined the numbers of bacteria.

The set of mixed broths were sub cultured into fresh broth for a second fitness assay. The broths were diluted 1:40 into fresh Middlebrook 7H9 broths (4 ml). These broths were incubated for a further 14 days and the numbers of bacteria determined again using a Miles and Misra plate count. In this way the fitness from 2 consecutive matched broths incubated for 6 and 14 days could be compared.

5.2.4 Relative fitness assay drug-resistant isolate (ET) and matched clinical drug susceptible strain (AT)

Broth cultures of the strains of *M. tuberculosis* (ET and AT) were diluted 1:400 into three fresh 4 ml Middlebrook 7H9 broth to create three mixed broths. These mixed broths were incubated at 37°C for 6 days. This set of mixed cultures containing both ET (MDR-TB) and AT (drug susceptible *M. tuberculosis*), was then used as the inoculum for a second set of broths. In this way both strains had been exposed to an identical environment.

The mixed broths containing both ET and AT clones were diluted 1:40 in fresh Middlebrook 7H9 broth (4ml). These broths were incubated at 37°C for 14 days. A Miles and Misra plate count determined the numbers of bacteria both at the start and end of the 14 days incubation period.

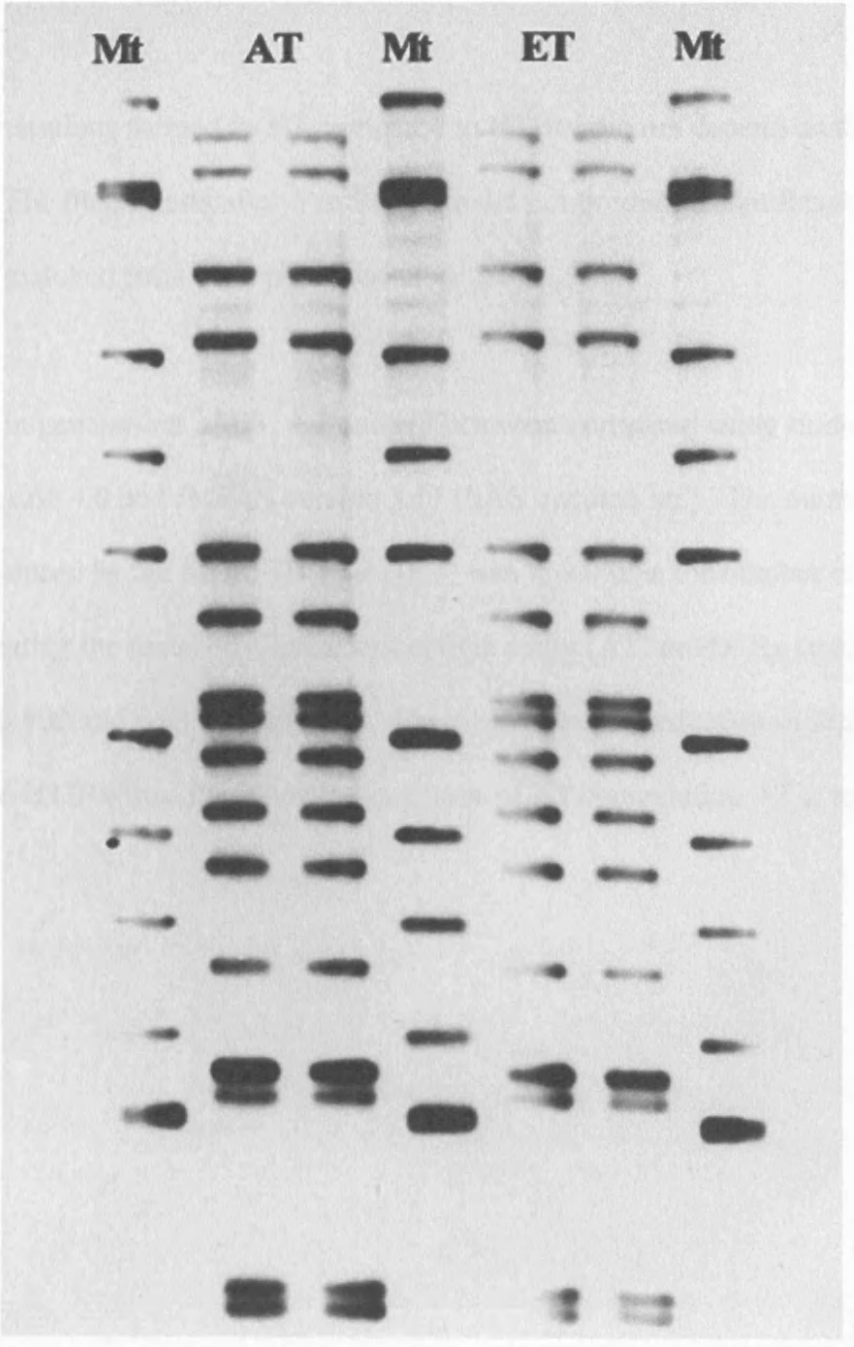
5.3 Results

Both strains were typed by A. Dickens (Department of Medical Microbiology Royal Free Hospital) using the international IS6110 method (Van Embden, Cave, Crawford et al. 1993) and PGRS technique (McHugh, Dickens & Gillespie 2000), and the patterns identified were identical (figure 5.1).

The number of generations formed in each mixed broth culture for ET and H37Rv and the calculated relative fitness of ET is shown in table 5.1. The mean relative fitness of ET compared to H37Rv was 0.52 with a standard deviation of 0.1.

The number of generations formed in each mixed broth culture for ET and AT and the calculated relative fitness of ET compare to AT are given in table 5.2

Figure 5.1 IS6110 RFLP pattern of the two patient strains AT and ET revealing the same pattern indicating a common ancestor. MT is the standard reference strain MT14323 (photograph supplied by A. Dickens)



The mechanism of drug resistance has not been elucidated for all of the antibiotics to which the strain ET became resistant. The *rpo*B gene of ET does however show a type B mutation PCR-SSCP as described in chapter 3, this indicates a mutation Ser 531⇒ Leu in the *rpo*B gene.

The ratio of generations formed by ET compared to H37Rv did not depend on the length of incubation. The fitness tests after 6 and 14 days did not produce a significantly different ratio (matched pairs t test p=0.5 see table 5.1).

The difference in generations of ET, AT and H37Rv were compared using students t test using Excel version 4.0 and JMP IN version 3.17 (SAS institute Inc). The number of generations produced by the MDR-TB strain (ET) was lower than the number of generations of either the matched clinical susceptible strain (AT) or H37Rv (matched pairs t test p = 0.002 and 0.03 respectively). There was a larger reduction in fitness of ET compared to H37Rv than the reduction in fitness of ET compared to AT (t test p =0.02).

Table 5.1 Relative fitness of ET compared to H37Rv

	Number of generations (6 day broth)		relative fitness (6 day broth)	Number of generations (14 day broth)		relative fitness (14 day broth)
ET	2.49	2.76	0.55	4.12	7.04	0.50
H37Rv	5.58	4.27		9.17	12.8	

Table 5.2 Relative fitness of ET compared to AT

	Number of generations formed in mixed broth culture.			Mean fitness (SD)
ET	9.07	8.14	6.81	0.73 (0.03)
AT	12.3	10.9	9.67	

5.4 Discussion

The initial infection of case ET was antibiotic sensitive but he complied with therapy poorly, attended for follow-up only intermittently, and multi-drug resistance emerged. Patient AT, the sister of patient ET, was infected prior to ET's infecting strain of *M. tuberculosis* developing multi-drug resistance. Like her brother AT complied with therapy poorly and attended follow-up erratically. The infection of AT however remained fully drug susceptible (Davies, Billington et al. 2000a). The close epidemiological link and identical IS6110 pattern indicate that the two isolates were recently derived from a common ancestor. Changes in fitness would therefore be associated with alterations in their environment including those caused by the acquisition of alleles for antibiotic resistance.

The drug resistant isolate (ET) has a relative fitness when compared to H37Rv of 0.52. The relative fitness for strain ET increases to 0.73 when compared to the drug susceptible clinical isolate AT. Direct comparison of H37Rv and AT, both isolates being drug susceptible, could not be performed. However, the higher relative fitness of ET compared to AT than H37Rv means that AT had a lower relative fitness than H37Rv in this test. Both the clinical isolates had a lower relative fitness than H37Rv measured *in vitro*.

H37Rv is a strain of tuberculosis isolated prior to the use of antimycobacterial agents. This has led to this strain being used internationally to control sensitivity tests. The isolate used in these experiments has been used to control susceptibility tests at the Royal Free Hospital for over 5 years. It is likely that H37Rv has adapted to artificial media and may show reduced clinical fitness. It is from such repeated subculture in artificial media that the vaccine strain of tuberculosis BCG was derived (Calmette & Guerin 1920). Thus caution must be observed as the relative fitness determined *in vitro* would not always reflect the position *in vivo*.

The isolate ET developed resistance to nine anti-tuberculosis agents. The mechanism of resistance has been elucidated for rifampicin. Rifampicin resistance was produced by a mutation in the *rpo*B gene which is associated with the lowest cost *in vitro* and is also the most common isolated clinically (Chapter 4). The lower relative fitness of ET compared to AT implies a physiological cost had been acquired by ET with drug resistance.

The physiological cost associated with drug resistance in the isolate ET may not be due to the alleles of resistance selected, but the forced selection of less optimal alleles at genes not related to drug resistance. The infection has undergone a series of genetic restrictions during the development of drug resistance. These restrictions must have reduced the population to a single cell, the original drug resistant mutant on acquiring each resistance gene.

Sampling error increases as populations reduce in size. A single poor allele sharing the chromosome with an essential gene such as drug resistance has more probability of rising to predominance from this reduced population. Clarke et al. (1993) working with an RNA virus, vesicular stomatitis virus found that large populations of virus increased their relative fitness on repeated subculture. Conversely cultures in which low virus numbers were passaged, causing severe genetic restriction, led to a reduction in relative fitness. This reduction in fitness was due to the accumulation of unfavourable mutations. In any population unfavourable mutations will arise. In small samples, such as one with continued restriction to a single cell, the probability of fixation of unfavourable mutants increases. This reduction has been described as Muller's ratchet (Andersson & Hughes 1996).

The strain ET has undergone a series of genetic restrictions. With the development of each resistance allele the population will have effectively been reduced to the single cell

that originally carried each resistant mutation. A set of nine genetic restrictions to a single cell over the three-year period could have produced the decline in fitness identified in this paper via Muller's ratchet.

It is possible that over a period of time the strain ET could adapt to produce the same relative fitness as the drug susceptible strain AT. Adaptation of drug resistant *E. coli* to the physiological cost of drug resistance has been studied *in vitro* (Schrag et al 1997). Compensatory mutations have been identified in *M. tuberculosis* in relation to isoniazid resistance (Sherman et al. 1996).

The multi-drug resistant isolate (ET) remained virulent and fully capable of causing progressive disease, the patient ET dying in 1999. Multi-drug resistant *M. tuberculosis* (MDR-TB) is pathogenic and fully capable of causing disease. The proportion of tuberculosis infection attributed to MDR-TB where anti-tuberculosis control programs have failed amply supports evidence for the pathogenicity of MDR-TB (Pablos-Mendez et al. 1998).

The fact that MDR-TB is capable of causing infection does not mean that it has an equivalent fitness to drug susceptible tuberculosis in the absence of antibiotic pressure. The reduced fitness identified *in vitro*, if reflected *in vivo* would mean that in the absence of antibiotic pressure MDR-TB would decline in competition with drug susceptible tuberculosis. The rate at which antibiotic resistance disappears would be

dependent on the probability of multiple infection by both resistant and sensitive strains of *M. tuberculosis.*

Chapter 6

An attempt to explore the adaptive landscape of the human host

6.1 Introduction

Bell (1997) states any character that evolves can only do so if it leads to a higher proportion of its progeny in subsequent generations. The selection of different alleles conferring the same phenotypic character, such as rifampicin resistance, is dependent on the relative fitness of the alleles (Billington et al. 1999).

Wright (1932) described the genome of a species as existing on an adaptive landscape. Wright, discussing the role of mutation and selection in evolution, described these mutant loci (which would now be referred to as alleles) as moving the genome within this adaptive landscape. A precise combination of alleles produces a specific phenotype, which interacts with the environment to produce a fitness value. This value correlates to a single position on the adaptive landscape. Is the fitness landscape for *M. tuberculosis* within the human host identical between hosts? An identical fitness landscape would indicate a single ideal sequence. A difference in landscape between hosts would require adaptation to each host and a range of ideal sequences.

This chapter explores the fitness landscape between individual hosts by comparing the fitness of isolates from three patients with MDR-TB. Three isolates from the source patient were compared, to measure the consistency of fitness estimates from a single

patient, and the fitness of three patient isolates were compared to identify any trends in relative fitness.

6.2 Method

6.2.1 Isolates

M. tuberculosis H37Rv (ATCC9360) was used as a control strain in the fitness assay.

Isolates from three patients involved in an MDR-TB outbreak in a London hospital were studied. As described by Breathnach et al. (1998) the strains had the same IS6110 RFLP pattern and epidemiological data indicated a common source for all the isolates. Three isolates were obtained from the source patient (patient S). Two isolates from the Royal Free Hospital and a third from the Brompton Hospital. A single isolate was used from the other two patients X and Y.

Sensitivity tests were performed on initial isolation for four of the five isolates. The isolates from the Royal Free Hospital had susceptibility tests performed at the Dulwich Hospital using the resistance ratio technique. The Brompton isolate had susceptibility tests carried out at the Brompton using the Bactec 460 and proportion technique. The sensitivity patterns for each of the isolates was identical for 9 antibiotics. Differences in susceptibility arose only in pyrazinamide and ciprofloxacin (see table 6.1). The ciprofloxacin and pyrazinamide susceptibility results were confirmed using the Bactec 460 and proportion technique at the Royal Free Hospital.

6.2.2 Ciprofloxacin susceptibility testing

The ciprofloxacin susceptibility test was performed using the Bactec 460 using the method recommended by the manufacturer.

Two Bactec 460 vials (pH 6.4) were prepared one containing 1ug/ml ciprofloxacin. A 0.5 McFarland broth culture of *M. tuberculosis* was prepared in Middlebrook 7H9 broth. This broth culture was diluted 1:40 in Middlebrook 7H9 broth and 0.1-ml of the dilute culture inoculated into the vial containing 1ug/ml ciprofloxacin. The broth culture was then diluted 1:100 in tween albumin broth and 0.1 ml of this dilution inoculated into the drug free vial.

The two vials were read daily and the growth index recorded. Susceptibility was recorded if on the day that the drug free control read greater than 30, the change on that day in the growth index for the drug-containing vial was less than the change in the control vial. Resistance was recorded if the change in the drug-containing vial was greater than the control vial, or the drug-containing vial had reached a growth index of 999.

A fully susceptible control H37Rv was set up with each batch of sensitivity tests to confirm activity of the antibiotic.

6.2.3 Pyrazinamide susceptibility testing

The pyrazinamide susceptibility test was performed using the Bactec 460 using the method recommended by the manufacturer. Two vials were prepared one containing 100-ug/ml pyrazinamide the second being drug free, both vials at a ph of 6.0. Equal size bacteria inocula were made into both vials. The vials were incubated at 37°C and read daily on the Bactec 460 and the growth values recorded. Resistance to pyrazinamide was recorded when the drug free vial read >200 if the drug vial was >11% of the drug free vial. Susceptibility was recorded if when the drug free vial was reading 200 the drug containing vial was <9% of the drug free vial. The test was repeated if the drug free vial failed to show a growth index of >200 in 10 days, or reach a growth index of >200 in less than 4 days. The test was also repeated if the drug vial was between 9 and 11% of the drug free vial when a growth index of 200 was reached in the drug free vial. A fully susceptible control H37Rv was set up with each batch of sensitivity tests to confirm activity of the antibiotic.

6.2.4 Pyrazinamidase gene PCR and sequencing

See section 2.6

6.2.5 *gyr*A PCR and sequencing

See section 2.5.

6.2.6 *gyr*B PCR and sequencing

See section 2.5.

6.2.7 Viable counts

Viable counts were performed by the technique of (Miles & Misra 1938) on rifampicin-containing and rifampicin-free plates. The dispersed broth was serially diluted 1:10 in tween-albumin broth (0.01% tween 80; Merck, Nuneaton, United Kingdom) and 0.02% bovine albumin (Sigma, Poole, United Kingdom) to form a series from 10^{-1} to 10^{-7}. The Tween albumin broth was vortexed briefly three times. Aliquots of these dilutions (50 uL) were inoculated onto Middlebrook 7H10 agar (Difco) containing either 5 mg/L rifampicin or no drug. The total colony count was estimated from the drug free media and the count of resistant cells was calculated from the drug containing plate. The number of susceptible colonies was calculated by subtracting the number of resistant colonies from the total.

6.2.8 Fitness assay

A mixed culture of MDR-TB isolate and H37Rv was prepared in a 4 ml 7H9 broth. The inocula were 100 uL of a 10-fold dilution of susceptible and 100 uL of a 100 fold dilution of MDR-TB from fully confluent broth cultures. The number of viable organisms was estimated, using the Miles and Misra plate count technique, at the beginning of incubation and after 2 weeks incubation at 37°C. The number of generations (G) could then be calculated with formula 6.1.

Formula 6.1 $G = (\log T_1 - \log T_0)/\text{Log } 2.$

Where T_1 is the final number of cells and T_0 is the initial number of cells.

The relative fitness of each isolate was calculated from the ratio generations of MDR-TB to susceptible H37Rv occurring in the same broth culture.

6.3 Statistics

The relative fitness of different isolates was compared using the one way ANOVA test. Differences between patients relative fitness values were confirmed using the students-t test and Tukey Kramer technique. All statistical tests were performed on JMP-IN (SAS Institute Inc).

6.4 Results

The ciprofloxacin susceptibility test indicated that both a single isolate from the source patient (S) and from patient X was ciprofloxacin resistant MIC (>4 ug/ml). No mutations were detected in the *gyr*B region but identical mutations were found in the *gyr*A gene (see table 6.4). This mutation (Asp 94 Gly) has been reported previously as leading to ciprofloxacin resistance in *M. tuberculosis* (Xu, Kreiswirth, Sreevatsan et al. 1996).

The pyrazinamide susceptibility data indicated that two of the strains 1S-RF from patient S and isolate Y from patient Y were resistant. There were no mutations identified in the pyrazinamidase gene.

The isolates from the source patient (patient S) had mean relative fitness values of 0.70, 0.86, and 0.84 (see table 6.2). There is no significant difference (one way ANOVA $p=0.34$) between fitness values and combined these produced a mean relative fitness value of 0.78 compared to H37Rv.

The relative fitness of the patient isolates S, X and Y are 0.78, 1.1 and 0.50 respectively compared to H37Rv (see table 6.3). There is a significant difference in the relative fitness values (one way ANOVA $p<0.001$). There is a significant difference between patient X, and Y and the source patient (S) using the t test and Tukey Kramer technique ($p<0.01$)

Table 6.1 Susceptibility test results of the isolates. Isolates S-1RF and S-B were from the source patient (S). Isolate X was from patient X and isolate Y from patient Y.

	Isolate S-1RF	Isolate S-B	Isolate X	Isolate Y
Streptomycin	S	S	S	S
Isoniazid	R	R	R	R
Rifampicin	R	R	R	R
Ethambutol	S	S	S	S
Ansamycin	R	R	R	R
Ethionamide	S	S	S	S
Capreomycin	S	S	S	S
Amikacin	S	S	S	S
Cycloserine	S	R	R	R
Pyrazinamide	S	R	S	R
Ciprofloxacin	S	R	R	S

Table 6.2 Relative fitness of three isolates from patient Z determined against H37Rv in Middlebrook 7H9 broth.

	Number of fitness assays	Mean relative fitness (standard deviation)
S-1RF	8	0.700 (0.228)
S-2RF	5	0.860 (0.193)
S-Brompton	4	0.84 (0.181)

Table 6.3 Relative fitness of isolates from the three patients S, X, and Y determined against H37Rv in Middlebrook 7H9 broth.

	Number of fitness assays	Mean relative fitness (standard deviation)
Patient S	17	0.778 (0.211)
Patient X	8	1.05 (0.163)
Patient Y	7	0.495 (0.122)

Table 6.4 Position of mutations in 4 of the 5 isolates detected in *pnc*A gene affecting pyrazinamide resistance, *rpo*B gene affecting rifampicin resistance, *gyr*A and *gyr*B gene affecting ciprofloxacin resistance. The initials WT indicate wild type and no mutations were identified.

Isolate	Gene *pnc*A	Gene *rpo*B	Gene *gyr*A	Gene *gyr*B
S-1	WT	Ser 531 Leu	WT	WT
S-B	WT	Ser 531 Leu	Asp 94 Gly	WT
X	WT	Ser 531 Leu	Asp 94 Gly	WT
Y	WT	Ser 531 Leu	WT	WT

6.5 Discussion

6.5.1 The effect of storage on isolates

When the temperature is above 0°C there is the possibility of growth, although at a reduced rate. Koch (1997) in a review of slow growth detailed the differences and similarities between slow and rapid growth. Koch described how slow growth is not just normal growth at a reduced rate. For example, DNA must be copied to RNA: this involves production of single stranded DNA which is very susceptible to mutagens. Copying of DNA must occur at the same rate as in normal growth, but longer periods between copying is then required. Furthermore the proteins produced in slow growth are different to those produced in normal growth. All of these aspects can be subject to genetic variation and so selection.

Extended periods on the same media, even with slow growth, must lead to a decline in nutrients and an increase in waste products. This will lead to competition for the nutrients and for resistance to waste products.

Storage of isolates of *M. tuberculosis* can provide a selective pressure and so lead to selection of alleles not predominate in the parent population. This variation in selective pressure during storage will result in different fitness values after storage, and between slopes stored in different environments. Two of the isolates from the source patient S-1RF and S-2RF were isolates identified isolated and stored at the Royal Free Hospital.

These isolates were stored at room temperature on LJ slopes. A third isolate from the source patient was isolated at the Brompton Hospital storage conditions are unknown.

There is no significant difference in the relative fitness of the three different isolates from the source patient (one way ANOVA p=0.34). This indicates variation during storage did not affect fitness of the source patient isolates.

6.5.2 The time of transmission of infection

Conventional epidemiological investigation basing the order on the time of the samples would indicate that patient X acquired infection before patient Y. However, isolate X from patient X was resistant to 5 drugs whilst the isolate from patient Y was resistant to only 4 of the 10 drugs tested. The increased resistances in isolate Y either indicates a later infection, when more resistance had been acquired, or that resistance developed in patient X. Comparison of resistance patterns and mutations may be used to further elucidate the order of transmission and where resistance arose.

Isolate S-1B was resistant to both ciprofloxacin and pyrazinamide. Isolate Y was susceptible to ciprofloxacin but resistant to pyrazinamide, and isolate X was resistant to ciprofloxacin and susceptible to pyrazinamide. Transmission of infection appears to have occurred prior to the double resistance arising in S-1B. But in which order did transmission occur, to patient X first and then patient Y or vice versa.

The mutation producing resistance in isolate Y and isolate S1-B is the same (see table 6.4). The common resistance allele in both the isolate S-1B and isolate X could indicate that patient X was infected only after ciprofloxacin resistance was developed. Resistance to ciprofloxacin was absent from isolate Y and this could indicate that patient Y was infected prior to patient X.

Ramaswamy & Musser (1998) in a review of antimycobacterial resistance mechanisms identified *gyr*A mutations as being more common than *gyr*B mutations in fluoroquinolone resistant *M. tuberculosis* . The specific mutation Asp94Gly isolated here is associated with a 60-fold increase in MIC, the largest increase reported by Xu, Kreiswirth, et al. (1996). The frequency of isolation of the different gyr*A* mutations is not reported.

Pyrazinamide resistance was absent from isolate X but present in both S1-B and isolate Y. The mutation leading to pyrazinamide resistance was not present in gene *pnc*A for either isolate S1-B or isolate. Pyrzinamidase activates pyrazinamide by converting it into the pyrazinoic acid. Mutations in the pyrazinamidase gene render the cell resistant by removing this activity. This is not however the only mechanism of pyrazinamide resistance. Davies et al. (2000b) found that 4 (21%) out of 19 *M. tuberculosis* isolates resistant to pyrazinamide using the Bactec 460 technique did not have a mutation in the *pncA* gene. The probability of two mutations outside the *pncA* gene occurring independently is therefore 4% (0.21x 0.21).

In order to deduce from the susceptibility data the order of infection, a greater understanding of the factors leading to the selection of resistance alleles and the probability of the same allele being independently isolated would need to be identified. The pattern of resistance can give an order of infection of patient X, then patient Y, if pyrazinamide is considered, or patient Y was infected then patient X if ciprofloxacin is considered. The order depends on the likelihood of both the specific mutations in the *gyr*A gene leading to ciprofloxacin resistance, the likelihood a mutation outside of the *pncA* gene leading to resistance to pyrazinamide, and the possibility of either mutation occurring independently on two separate occasions.

6.5.3 Evidence of adaptation to the host

The relative fitness of the *M. tuberculosis* isolate affecting patient X (1.1) is higher than the relative fitness of the isolates affecting the source patient S (0.78). The relative fitness of the *M. tuberculosis* isolate affecting patient Y (0.50) is lower than the isolate affecting the source patient.

The artificial media in which the relative fitness of the three patient's clones were measured was not the environment to which these strains had adapted. The measured alteration in relative fitness, whilst it reflects a change, need not, reflect the direction in which the change had occurred within the patient. An improvement in relative fitness *in vitro* does not necessarily lead to an improvement in relative fitness *in vivo*. However this is evidence for a genetic difference between isolates.

Evolution, the inherited change in gene frequency distribution, can occur either through selection, indicating changes are of benefit, or through genetic drift. Genetic drift is the random accumulation of genetic alterations, which occurs purely from chance with no beneficial effect. The *M. tuberculosis* isolates of both the infected patients X and Y altered from the source patient isolates' relative fitness. This alteration in the relative fitness indicates an alteration in the genome.

Genetic change was too frequent to be by chance, or genetic drift. This indicates selective pressures were great enough to lead to the emergence of adaptive changes in *M. tuberculosis* gene sequence in the isolates from patients X and Y. That there was an observable difference in the genetic fitness of these isolates as compared to the parent indicates that the adaptive landscapes of patients X and Y were significantly different to S.

Compensatory mutations, which reduce or eliminate the relative fitness reduction created by antibiotic resistance, must compete along with other unknown adaptive mutations specific to the individual host.

The Government's report to the House of Lords' Select Committee on Science and Technology on antibiotic resistance entitled 'The path of least resistance' (Department of Health 1998b) asked the pertinent question 'is antibiotic resistance a one way street?'. Is retention of antibiotic resistance dependent on the selective pressure of antibiotic usage? The answers, it would appear, are at least in part dependent on the fitness cost

associated with antibiotic resistance, the possibility of compensatory mutations reducing this cost and the relative cost of adaptation to each new host.

Chapter 7

The role of selection and genetic drift in the evolution of drug resistance

Drug resistance in *M. tuberculosis* only arises as a result of mutation and selection. The necessity of generating variation by mutation is amply illustrated by the history of the development of chemotherapy. Single therapy has been effective where low numbers of bacilli are present such as the continuation phase of therapy. Triple therapy is however essential where numerous bacilli are present such as in pulmonary tuberculosis (Chan & Iseman 2002). The necessity for generating genetic variation emphasises the importance of the mutation rate.

One prediction of such variation is that it can lead to genetic drift. Genetic drift is the adoption of mutations or alleles not due to any advantage but due to chance alone (Bell 1997). The forced adoption of a rare but essential mutation such as antibiotic resistance would enhance the role of genetic drift. If advantageous alleles were not rare and there is a wide amount of genetic variation, then the role of selection would be enhanced.

There is evidence of genetic variation in *M. tuberculosis* during infection. De Boer, Kremer, Borgdorff et al. (2000) examined the IS6110 restriction fragment length polymorphism banding pattern (RFLP) of 1,277 isolates of *M. tuberculosis* for low intensity bands. Isolates with identical IS6110 RFLP patterns are considered to have originated from the same ancestral strain. Multiple low intensity banding can indicate multiple infection with two or more strains of *M. tuberculosis* (Pavlic, Allerberger,

Dierich et al. 1999). De Boer et al (2000) identified no multiple low intensity bands in the 1,277 isolates but identified 94 (7.4%) single low intensity bands. 6 single colonies picked from cultures with low intensity bands revealed the culture to consist of two populations with the RFLP differing by the single band. Low intensity banding patterns were produced when there was a population differing by a single band and this population made up 10% to 30% of the DNA used to produce the RFLP. This indicates large sub-populations of *M. tuberculosis* can exist in clinical infections

The frequency of antibiotic resistance isolated *in vivo* in *M. tuberculosis* as noted in chapter 3 is not reflected in the measured mutation rate (table 3.1). The predominance of one allele of rifampicin resistance Ser 531 to Leu which accounted for 42% of clinical isolates with identified rifampicin mutations reported by Ramaswamy & Musser (1998) is not explained by a raised mutation rate for that allele (chapter 3). That allele was however associated with a low fitness deficit measured *in vitro* (chapter 4). Wichelhaus, Boddinghaus, Besier et al. (2002) found the same association between fitness and the allele of rifampicin resistance in *S. aureus*. The higher fitness means that that allele is both more likely to be present and is more likely to outcompete less fit resistant alleles and so come to predominance (see 1.13). The association with fitness and emergence of resistant alleles is not restricted to rifampicin.

Pym, Saint-Joanis & Cole (2002) studied the fitness of strains of isoniazid resistant mutants *in vivo*. The mutation S315T in the *kat*G gene leads to clinically significant resistance to isoniazid (5ug/ml) with an active catalase gene product, the reduction in

activity was about 12%. This mutant is the most commonly clinically isolated isoniazid resistant allele (Ramaswamy & Musser 1998). Pym et al. (2002) injected mice with *M. tuberculosis* both deficient in *kat*G with normal *kat*G activity and with the mutation S315T. The growth of bacilli with the mutation S315T in mice lungs and spleen over a forty-day period after injection was larger than that of the *kat*G deficient strains approaching if not equalling that of the wild type strains. This supports the hypothesis that the mutation S315T mutation is selected not only due to the resistance to isoniazid conferred, but its catalase activity is needed to detoxify host antibacterial radicals. S315T has a higher fitness *in vivo* due to this ability to neutralise the oxidative burst of host macrophage and other such antibacterial radicals.

Evidence that streptomycin leads to growth rate inhibition due to a reduced rate of protein synthesis, and that adaptive mutations can then ameliorate that fitness deficit was supplied by Schrag, Perrot & Levin (1997) and is described in the introduction (section 1.15). In all the cases described, the allele of resistance selected most frequently produces the least fitness deficit.

Antibiotic control programs are based on the theory that there is a fitness deficit associated with antibiotic resistance (Anderson 1999). In chapter 5 a fitness deficit associated with an isolate of MDR-TB was identified compared to a closely related fully susceptible isolate. These isolates sharing a close common ancestry will share a common genomic heritage, the difference in fitness reflecting recent genomic changes. This does not mean the isolates were isogenic differing only in terms of specific drug

resistance mutations. The genomic history of clinical isolates is unknown. Mutations other than drug resistance may have been present either from adaptation to host environments or from drift or founder effects. Mutations sharing the genome with essential mutations, such as drug resistance, can be selected for due to the essential allele's presence (Bell 1997). A number of matched clinical isolates, drug resistant and susceptible, would need to be tested to overcome this uncertainty in the history of clinical isolates.

Ordway et al. (1995) measured the growth rate of 15 clinical isolates in mice. This included 3 MDR-TB isolates (defined as resistant to isoniazid and rifampicin). Growth rates were compared to that of a laboratory control strain, the Erdman strain. No correlation with antibiotic resistance and growth rate was identified. These isolates were not related and so revealed that heterogeneity in *M. tuberculosis* isolates is a larger factor in variance in growth rate than antibiotic resistance.

Cohen et al (2003) reviewed population-based evidence for a reduction in fitness associated with drug resistance. The evidence was that fitness estimates of *M. tuberculosis* are very variable. Cluster analysis was highly dependent on both the population studied and the size of the study. Both laboratory and population studies do reveal that heterogeneity in *M. tuberculosis* growth and infectivity does exist. This indicates that *M. tuberculosis* is currently evolving in areas other than drug resistance.

The heterogeneity in growth rates of *M. tuberculosis* raises the question: what is the fitness landscape of the human host? Is the landscape a single peaked landscape with a single ideal *M. tuberculosis* sequence or a multiple peaked landscape indicating a range of different ideal genomes?

In chapter 6 evidence is produced that there is variation in the fitness of isolates between patients. This alteration in fitness was apparent with none of the three isolates from a multi-drug resistant tuberculosis outbreak sharing the same fitness. This high amount of variation indicates there is some alteration in the genome with the transfer of infection between patients.

Musser, Amin & Ramaswamy (2000) found little genetic change in cell wall components. Identifying the reason for the alteration in fitness between patient isolates may reveal more about the adaptive necessities of *M. tuberculosis* to the host and those genes where selective pressures are produced by the host.

Kato-Maeda, Rhee, Gingeras et al. (2001) identified wide variation in the genome of clinical isolates of *M. tuberculosis*. The genome of 19 isolates of *M. tuberculosis* was compared to that of H37Rv and H37Ra. Each isolate was missing an average 0.3% (13,248 bp) of the genome present in H37Rv. This correlates to some or all of 17.2 open reading frames with no isolate having more than 38 open reading frames deleted. Due to the method used, DNA sequences absent from H37Rv but present in the clinical isolates could not be detected. No evidence was found for variation during chains of infection,

no change being detected in three isolates from a recognised chain of infection. However the method used could not detect deletions shorter than 350 bp. This indicates that the heterogeneity detected in fitness estimates between unrelated *M. tuberculosis* isolates, is reflected in wide variation in the genome contents. However the variation in fitness found between isolates in chain of infection relies on more subtle causes than the 350 bp deletions Kato-Maeda et al. (2001) could detect.

The presence of wide-spread strains and clades of *M. tuberculosis* such as the Beijing strains have been identified indicating a range of infectivity in *M. tuberculosis* (Filliol, Driscoll, van Soolingen et al. 2003). Analysis of the IS6610 patterns of *M. tuberculosis* isolates collected in London has revealed that patients were more likely to be infected with strains of tuberculosis related to their racial origin (Maguire et al. 2002). Hirsh, Tsolaki, DeRemier et al. (2004) examined the genetic relationship of 100 *M. tuberculosis* isolates using microarrays to identify deletions present in the clinical isolates but absent from H37Rv. All of the clinical isolates were from separate genetic lineages collected over 12 years in San Francisco. Molecular phylogenetic analysis of the deletions revealed the isolates related most closely to the racial origin of the patient. Four large geographic regions, East Asia, the Philippines, Americas, and a group consisting of African, European and Middle Eastern origin were identified.

Hirsh et al. (2004) identified that the population of infection between Philippine and East Asian tuberculosis separated between 240 and 1000 years ago. This separation of infection into human racial groups over such a prolonged period led Hirsh at al. (2004)

to speculate on the possibility 'of adaptation by specific *M. tuberculosis* lineages to the genetic cultural or environmental characteristics of particular populations of hosts'.

The heterogeneity in tuberculosis growth rates and genome structure raises the possibility that antibiotic use is not as great a selective pressure as it would appear. Methicillin resistant *Staphylococcus aureus* has increased greatly over the last decade. Enright, Robinson, Randle et al. (2002) identified methicillin resistance as repeatedly emerging from successful clones of methicillin susceptible clones of *Staphylococcus aureus*. It is the combination of drug resistance and strains well adapted to transmission in hospitals that produce epidemic MRSA strains.

Antibiotic resistance is a vital character for treatment of infection. It is however only one component of the infective repertoire of the infecting organism. The ease with which antibiotic resistance is measured and the importance of this character for treatment has lead to this being emphasised. However, the combination of drug resistance and infectivity may be required to produce an epidemic MDR-TB strain.

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Appendix A

Media

Tween albumin broth.

0.2% bovine fatty acid free albumin

0.01% Tween 80

Dissolve in deionised water and filter sterilised.

Middlebrook 7H9 Broth

4.7g Middlebrook 7H9 powder (Difco)

2 ml Tween 80

900 ml deionised water.

ADC supplement, Albumin, Dextrose, Catalase solution (Difco).

Autoclave in 180 ml aliquotes. To use add 20 ml ADC supplement (Difco) to 180 ml broth and distribute using sterile technique.

Middlebrook 7H10 Agar.

1.9g 7H10 agar powder (Difco)

0.5 ml Glycerol

90 ml deionised water.

OADC supplement, Oleic acid, Albumin, Dextrose, Catalase solution (Difco).

Autocalve and cool to 50 C in a water bath. Add 10 ml OADC supplement (Difco) and any desired antibiotic. Pour molten agar in 20 ml aliquotes into petri dishes.

Appendix B

Papers published as a result of the research carried out for this thesis

Billington, O. J., McHugh, T. D., & Gillespie, S. H. 1999, "Physiological cost of rifampin resistance induced in vitro in *Mycobacterium tuberculosis*", *Antimicrob.Agents Chemother.*, vol. 43,no. 8, pp. 1866-1869.

Davies, A. P., Billington, O. J., Bannister, B. A., Weir, W. R., McHugh, T. D., & Gillespie, S. H. 2000a, "Comparison of fitness of two isolates of *Mycobacterium tuberculosis*, one of which had developed multi-drug resistance during the course of treatment", *J.Infect.*, vol. 41,no. 2, pp. 184-187.

Gillespie, S. H., Billington, O. J., Breathnach, A., & McHugh, T. D. 2002, "Multiple drug-resistant *Mycobacterium tuberculosis*: evidence for changing fitness following passage through human hosts", *Microb.Drug Resist.*, vol. 8,no. 4, pp. 273-279.

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0066-4804/99/$04.00+0

Physiological Cost of Rifampin Resistance Induced In Vitro in *Mycobacterium tuberculosis*

O. J. BILLINGTON, T. D. McHUGH, AND S. H. GILLESPIE*

Journal of Infection (2000) 41, 1–4
doi:10.1053/jinf.2000.0711, available online at http://www.idealibrary.com on IDEAL®

CASE REPORTS

Comparison of Fitness of Two Isolates of *Mycobacterium tuberculosis*, one of Which had Developed Multi-drug Resistance During the Course of Treatment

A. P. Davies[1], O. J. Billington[1], B. A. Bannister[2], W. R. C. Weir[2], T. D. McHugh[1] and S. H. Gillespie[1]*

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MICROBIAL DRUG RESISTANCE
Volume 8, Number 4, 2002

Multiple Drug-Resistant *Mycobacterium tuberculosis:* Evidence for Changing Fitness Following Passage Through Human Hosts

STEPHEN H. GILLESPIE,[1] OWEN J. BILLINGTON,[1] AODHAN BREATHNACH,[2] and TIMOTHY D. McHUGH[1]

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